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METABOLISM OF THE STEROID HORMONES— THE METABOLISM OF PROGESTERONE AND ETHYNYL TESTOSTERONE[1]

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THIS INVESTIGATION is concerned with the possible conversion of ethynyl testosterone to pregnanediol-3(α),20(α), the question of absorption of progesterone from the gastrointestinal tract, and the conversion of progesterone to pregnanol-3(α)-one-20.

Allen, Viegiver and Soule (1944) reported the excretion of a conjugated steroid in the urine of patients suffering from secondary amenorrhea who had received ethynyl testosterone. Pregnanediol-3(α),20(α) was not actually identified but the authors suggested that the isolated material contained pregnanediol-3(α),20(α) or a related steroid. Other workers have been unable to demonstrate the presence of pregnanediol-3(α),20(α) after the administration of ethynyl testosterone (Goldberg and Hardegger, 1941; Hamblen, Cuyler and Hirst, 1940).

During the course of our investigations on the absorption of progesterone from the gastrointestinal tract a paper by Masson and Hoffman (1945) appeared which demonstrated that progesterone may be absorbed from the gut in the rabbit. Our studies on the absorption of progesterone from the gastro-intestinal tract were done in men.

SUBJECTS AND MATERIALS

The three subjects selected for these studies consisted of a thirty-two-year-old man (A. G.), who was suffering from Addison's disease

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(Shipley, 1944), a seventy-one-year-old man (M. A.) who was diabetic and showed signs of hypopituitarism, and a 26-year-old woman (H. I.) suffering from secondary amenorrhea of about two years' duration.

Progesterone and ethynyl testosterone[2] were administered orally in capsules and the daily dose was usually administered in two equal doses in the morning and evening.

TABLE 1. THE EXCRETION OF PREGNANEDIOL AFTER THE ORAL ADMINISTRATION OF PROGESTERONE

Patient	Clinical status	Experimental day	Amount of progesterone administered orally mg.	Pregnanediol-3(α) 20(α)[1] excreted in the urine mg./24 hrs.
A. G. (Male)	Addison's Disease	1	0	0
		2	0	0
		3	150	6.7
		4	0	0
		5	0	0
		6	300	21.1
		7	0	0
		8	0	1.6
M. A. (Male)	Diabetes	1	0	0
		2	0	0
		3	300	22.4
		4	0	13.7
		5	0	17.6
		6	0	1.3
		13	300	8.0[2]
		14	0	14.9
		15	0	10.1
		18	300	30.6
		19	300	45.0
		20	0	22.4
		21	0	13.9
		22	0	3.5
		23	0	1.6

[1] A portion of this was found to be pregnanol-3(α)-one-3 as described in the section, Conversion of Progesterone to Pregnanol-3(α)-one-20.
[2] Sixteen-hour sample.

The assays for pregnanediol glucuronide were done by the method of Venning (1938).

Studies on Ethynyl Testosterone

Ethynyl testosterone in total amounts of 600 to 1200 mg. was administered orally to all three of the subjects, A. G., M. A., and H. I. No pregnanediol glucuronide could be demonstrated in any of the urines collected after the administration of ethynyl testosterone.

[2] We are indebted to Ciba Pharmaceutical Products, Inc., for a generous supply of both steroids.

Absorption of Progesterone

The gastrointestinal absorption of progesterone was studied in two male patients A. G. and M. A. Table 1 summarizes the experimental procedure and the results obtained. The percentage conversion of progesterone to pregnanediol appears to be lower in the Addison's disease patient than in the diabetic-hypopituitary patient. Thus in the Addison's disease patient the administration of 150 mg. of progesterone yielded only 4.5% pregnanediol, while 300 mg. of the hormone resulted in an excretion of pregnanediol only to the extent of 7.0%. In the second patient the administration of 300 mg. of progesterone per day resulted in pregnanediol excretions of 18.3% and 11.0% respectively. When 600 mg. of progesterone was administered, that is, 300 mg. per day for two days, the recovery for the period was 19.5%. Although the excretion product has been described as pregnanediol, it was demonstrated that a portion (approximately 10%) of the isolated product was pregnanol-3(α)-one-20.

Conversion of Progesterone to Pregnanol-3(α)-one-20.

The total amount of material designated as sodium pregnanediol glucuronide isolated from the urine of M. A. after the administration of progesterone was combined and amounted to 205 mg. of pregnanediol equivalent. This material was refluxed for six hours in a mixture of 200 cc. of distilled water, 30 cc. of concentrated hydrochloric acid and 300 cc. of benzene. The mixture was cooled, the benzene separated in a separatory funnel and the water layer reextracted twice more with 100 cc. portions of benzene. The combined benzene extracts were washed with water and evaporated to dryness in vacuo. The dry residue was treated with Girard reagent T and separated into the ketonic and non-ketonic fractions as previously described (Dorfman, Cook and Hamilton, 1939). The ketonic fraction yielded a crystalline material which after three recrystallizations from 90% methanol yielded a compound which melted at 143-146.5°C.[3] (weight 22 mg). This compound was mixed with an authentic sample of pregnanol-3(α)-one-20. (M. P. 145–148°C.),[4] and the melting point of the mixture was 144–146°C. An acetate was made which melted at 95–97°C. and when mixed with an authentic sample of pregnanol-3(α)-one-20 acetate (M. P. 96–97°C.) the melting point was 95.5–97°C.

DISCUSSION

Contrary to the findings of Allen *et al* (1944) we have been unable to demonstrate the presence of a steroid conjugate after the administration of ethynyl testosterone.

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[3] All melting points were taken with the Fischer-Johns apparatus and are uncorrected.

[4] We are indebted to Dr. K. Dobriner of Memorial Hospital for this sample.

There appears to be little doubt that progesterone is absorbed from the gastrointestinal tract. The recovery of as much as 18.3 and 19.5% of the progesterone as pregnanediol equivalent from the urine of one of our patients indicates that a good portion of the hormone was absorbed. However, these calculations are based on the assumption that the glucuronide isolated by the Venning procedure was pure pregnanediol. However, it has been shown that at least in one case the so-called pregnanediol glucuronide was not pure but actually contained pregnanol-3(α)-one-20 as an impurity. Thus if the yields are recalculated on the basis that progesterone was converted to both pregnanediol-3(α),20(α) and pregnanol-3(α)-one-20 it is found that 1.8% of the administered progesterone appeared as pregnanol-3(α)-one-20 and approximately 15.4% as pregnanediol-3(α),20(α).

The finding of pregnanol-3(α)-one-20 as a contaminant of the pregnanediol glucuronide preparation in this study is similar to the findings of Marrian and Gough (1946) who were able to demonstrate that so-called pure samples of pregnanediol glucuronide isolated from human pregnancy urines contained appreciable quantities of a water soluble complex of pregnanol-3(α)-one-20.

SUMMARY

It has not been possible to demonstrate the conversion of ethynyl testosterone to pregnanediol-3(α),20(α) or any other steroid either in men or in a woman suffering from secondary amenorrhea when daily oral doses of 300 mg. of the steroid were administered.

Progesterone absorption from the gastrointestinal tract was demonstrated by the isolation of sodium pregnanediol glucuronide after the administration of the progestational hormone. However, it was shown that the pregnanediol complex isolated by the Venning procedure contained a small amount of pregnanol-3(α)-one-20 in addition to pregnanediol-3(α),20(α). Thus it appears that pregnanol-3(α)-one-20 can be classed as a metabolite of progesterone along with pregnanediol-3(α),20(α).

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METABOLISM OF THE STEROID HORMONES— THE CONVERSION OF ISOANDROSTERONE TO ANDROSTERONE[1]

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THE FACT that the adrenal cortex contributes to the metabolic pool of androgens in the body and the 17-ketosteroids in the urine is well known. Hirschmann (1941) was able to isolate androsterone and etiocholanol-3(α)-one-17 from the urine of ovariectomized women in concentrations almost equivalent to that isolated from the urine of normal women. In Addison's disease the urinary titer of androgens and 17-ketosteroids is significantly reduced. In patients with adrenal cortical hyperplasia or tumors, the concentration of androgens and 17-ketosteroids is increased.

Although the evidence indicates that some of the androgens found in normal urine were derived from the adrenal cortical steroids, little is known of the mechanism of the conversion of these steroids to major androgen metabolites, such as androsterone and etiocholanol-3(α)-one-17. Of the three C-19 compounds which have been isolated from adrenal tissue, one, Δ^4-androstenedione-3,17, has been shown to yield androsterone and etiocholanol-3(α)-one-17 after administration to men (Dorfman, Wise, Shipley, 1947). However, it is to be remembered that Reichstein, who isolated these compounds from adrenal tissue, has indicated that these C–19 compounds may be artifacts.

Since a large number of compounds of the adrenal cortex have been shown to have the 3(β)-hydroxy-allo configuration in rings A and B and since only a small portion of the urinary steroids have this configuration, there appeared a good likelihood that compounds having this configuration may be further metabolized. Due to the fact that compounds of the adrenal cortical series having 21 carbon atoms were not available for metabolism studies, the model steroid isoandrosterone was investigated.

EXPERIMENTAL

The patient was a fifty-one-year-old man showing the symptoms of the Laurence-Moon-Beidl syndrome described elsewhere in de-

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tail (Case 3) (Roth, 1946). The chief reason for choosing this subject was the fact that the individual has exhibited hypogonadism during his entire adult life. A control sample of urine collected over a thirteen-day period showed a 17-ketosteroid value of 3.2 mg. a day in the α-ketonic neutral fraction while the β-ketonic neutral fraction showed a titer of less than 0.05 mg. per day.

During the experimental period the patient received seven daily injections of 300 mg. of isoandrosterone acetate[2] contained in 6 cc. of sesame oil and an eighth injection of 40 mg. of isoandrosterone acetate. Urine was collected during the injection period and for the following two days.

The urines, both the control and experimental samples, were extracted daily after the completion of the twenty-four-hour period. For the extraction the urine was acidified with 15% by volume of concentrated hydrochloric acid and refluxed for ten minutes. The acidified hydrolyzed urine was cooled, 10 grams of sodium chloride was added per 100 cc. of urine and the mixture extracted with one-quarter volume of benzene. The benzene was separated and the extraction process repeated twice more. The combined benzene extracts were evaporated to dryness, taken up in ether and the ketonic neutral fraction prepared as previously described (Dorfman, Cook, and Hamilton, 1939). The nonketonic fraction was reserved for future isolation studies. The ketonic neutral fractions of both the control urine and the experimental urine were subjected to partition with digitonin, and the digitonin insoluble ketonic fractions and digitonin soluble ketonic fractions were prepared.

ISOLATION OF ISOANDROSTERONE: the digitonin precipitable ketonic fraction, which amounted to 0.2 gms., was adsorbed on a column of 5.0 gms. of aluminum oxide (Merck's Brockmann) from a 5 cc. solution of carbon tetrachloride. The column was eluted twice with carbon tetrachloride followed by carbon tetrachloride containing increasing concentrations of absolute ethanol ranging from 0.1% to 0.3% absolute ethanol in carbon tetrachloride, there was obtained 143 mg. of crystalline material, after recrystallization from methanol, which melted[3] at 173–174°C. A mixture of the crystalline material with an authentic sample of isoandrosterone (M. P. −172–3°C.) melted at 173–4°C. An acetate was prepared with pyridine and acetic anhydride which after recrystallization melted at 102.5–103°C. When mixed with an authentic sample of isoandrosterone acetate (M. P. 114.5–115.5°) the melting point was 112–113°C.

ISOLATION OF $\Delta^{2 \text{ or } 3}$-ANDROSTERONE-17 and ANDROSTERONE: The ketonic fraction which was not precipitated with digi-

[2] We are indebted to Ciba Pharmaceutical Products, Inc., for the generous supply of isoandrosterone acetate.

[3] All melting points were taken with the Fischer-Johns apparatus and are uncorrected.

tonin amounted to 0.4 gms. It was subjected to chromatographic analysis using 12 g. of aluminum oxide. The urinary fraction was dissolved in 25 cc. of carbon tetrachloride and adsorbed on the aluminum oxide. Elutions were performed with 100 cc. portions of carbon tetrachloride and carbon tetrachloride containing 0.1% to 0.3% ethanol.

Elution with carbon tetrachloride (fraction 2) yielded 125 mg. of crystalline material which melted at 96–98°C. After four further recrystallizations from methanol the melting point was raised to 102–103°C. An oxime was prepared which melted at 152–153°C. When mixed with an authentic sample of $\Delta^{2 \text{ or } 3}$ androsterone-17 oxime (M. P. 151–2°C.) the melting point was 151–2°C.

One hundred and fifty-one milligrams of crystalline material was obtained from fractions 5, 6, 7, 8, which melted at 181–3°C. after recrystallization from methanol. When mixed with an authentic sample of androsterone (M.P. 181°C.) the melting point was 182–183°C. An acetate was prepared which melted at 162.5–163.5°C. When mixed with an authentic sample of androsterone acetate M.P. 160–160.5°C. the melting point was 160–161°C.

DISCUSSION AND CONCLUSIONS

The fact that isoandrosterone may be converted to and excreted as androsterone indicates a possible reason for the relatively high concentration of 3(β)-hydroxy-allo compounds in the adrenal gland and the relatively low concentrations of these steroids in urine. In the metabolism of the adrenal cortical steroids to 17-ketosteroids, there still remains the consideration of the conversion of the C–21 steroids to the 17-ketosteroids. However, if this reaction does take place it can be seen that the 3-(β)-hydroxy group can be inverted to the 3(α) hydroxy configuration.

A likely mechanism for this inversion of the 3-hydroxyl group is by way of the 3-ketone with the subsequent reduction of the 3-ketone principally to the 3(α) form. That such a reaction occurs is seen from the fact that after the administration of androstanedione −3, 17 the bulk of the 17-ketosteroids excreted is recovered as androsterone.

Seven and six-tenths per cent of the administered isoandrosterone was recovered from the urine as such while 8.1% appeared as androsterone. An additional 6.7% of the administered isoandrosterone appeared as $\Delta^{2 \text{ or } 3}$-androsterone-17. The latter compound may well be derived, at least in part, from androsterone. Thus the reported per cent of isoandrosterone (8.1) to androsterone is a minimum estimate.

SUMMARY

The administration of isoandrosterone to a hypogonadal man showing the signs of the Laurence-Moon-Beidl syndrome gave rise to the excretion of androsterone (8.1%) in addition to some unchanged

isoandrosterone (7.6%). An additional 6.7% of the administered isoandrosterone was recovered as $\Delta^{2 \text{ or } 3}$-androstenone-17. The latter compound may well be derived, at least in part, from androsterone.

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STUDIES ON THE BIOASSAY OF HORMONES— THE ASSAY OF ESTROGENS BY A CHICK OVIDUCT METHOD

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THIS COMMUNICATION is concerned with the response of the chick oviduct to various estrogens, particularly to the view of using this endpoint as a method of assaying estrogens.

ANIMALS, MATERIALS, METHODS

For these studies White Leghorn Pullets were employed which were obtained from Kerr Chickeries in Frenchtown, N. J. The animals were received in Cleveland between one to three days after hatching and immediately placed on a diet of commercial chick starting mash. The bulk of the experiments were started on the fourth day of life although some were started on the seventh day of life. In either case the material to be studied was injected subcutaneously once daily for five days and the determination of oviduct weight and body weight was done at autopsy 24 hours after the last injection. The total material administered per animal was dissolved in 0.5 cc. of corn oil and 0.1 cc. was injected daily. The unstimulated control group of animals received a similar schedule of treatment with only the corn oil.

The animals were killed with chloroform and the oviduct carefully dissected, blotted on a cloth towel and weighed immediately to 0.5 milligram on a torsion balance. All results are expressed as 100 times the ratio of weight of the oviduct in milligrams to the body weight in grams.

The estrogens studies consisted of stilbestrol, estrone, estradiol benzoate, estradiol, and methoxy bisdehydro doisynolic acid (MDDA).[1]

The method of studying the precision of the assay technique consisted in assaying an estrogen against itself using the design previously described by Bliss (1944). Thus the following experimental design was employed:

$$\frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown}}$$

and

$$\text{Standard} = \text{Unknown}$$

Using this design it was possible to easily ascertain the potency ratio, the error range of the potency ratio, and the significancy of the difference of the slopes.

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[1] All of the estrogens used, with the exception of stilbestrol, were generously supplied by Ciba Pharmaceutical Products, Inc. The stilbestrol was supplied by Winthrop Chemical Co., Inc.

RESULTS

Table 1 illustrates the crude data of the response of the oviduct to the various estrogens when the animals were autopsied on the 9th day of life.

In Table 1 we have 5 groups of animals which received no estrogen but did receive the usual amount of pure corn oil. The mean oviduct ratio varied from 9 ± 0.86 to 11 ± 0.87 for groups of animals which varied in number from 15 to 25. Stilbestrol which was the most active estrogen studied still showed a steeply rising curve when 1600 μg. total dose was administered. Thus the maximum dose level has not as yet been reached. However, at this level of 1600μg. of stilbestrol the mean oviduct ratio was 56 times as great as that of the controls.

TABLE 1. OVIDUCT RESPONSE TO ESTROGENS (9TH DAY)

Substance	Total amount admin. μg.	Number of chicks	Oviduct ratio ±S.E.
0	0	15	11 ± 0.62
0	0	21	11 ± 0.87
0	0	25	10 ± 0.49
0	0	24	11 ± 0.43
0	0	17	9 ± 0.86
Stilbestrol	25	28	29 ± 1.8
	50	24	55 ± 2.3
	100	54	71 ± 2.4
	200	53	143 ± 6.3
	400	74	270 ±10.3
	800	19	464 ±29.4
	1600	18	564 ±26.6
Estrone	2.5	16	12 ± 0.8
	10.0	21	14 ± 0.8
	40.0	18	16 ± 0.9
	160.0	52	20 ± 1.0
	640.0	68	31 ± 1.0
	1280.0	40	75 ± 1.1
Estradiol Benzoate	25	25	10 ± 0.5
	50	25	16 ± 0.9
	100	44	22 ± 1.1
	200	42	59 ± 3.8
	400	23	123 ± 8.6
Estradiol	100	34	26 ± 0.9
	200	30	33 ± 1.2
	400	52	41 ± 1.4
	800	21	69 ± 4.2
	1600	23	105 ± 7.1
MDDA	25	19	12 ± 0.8
	100	18	17 ± 3.0
	400	42	101 ±28
	1600	45	108 ±33

The response of the oviduct to MDDA is of interest from two points of view. First, although a reasonably good response of the oviduct was found at the level of 400 μg., increasing the dose to 1600

μg. caused no real increment in oviduct weight; a change from a ratio of 101 to that of 108. Secondly, as indicated by the standard error of the mean the variations of the responses within a group particularly at the levels of 400 and 1600 μg. are unusually great.

The Assay of Estrone

Tables 2 and 3 illustrate the use of the assay method for estrone. Table 2 is concerned with the data collected for the assays ending on the 9th day of life of the chicks while Table 3 is concerned with the data for the autopsies performed on the 16th day.

TABLE 2. ASSAY OF ESTRONE (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{1280\ \mu\text{g.}}{640\ \mu\text{g.}}$$

N	b	s	t	λ	Pot. Ratio ±S.E. %
5	150	8.87	0.531	0.059	83 ± 6
	156	16.3	1.542	0.104	117 ± 13
	127	18.9	1.567	0.149	127 ± 19
	154	25.6	0.228	0.161	107 ± 18
7	112	12.0	0.253	0.107	88 ± 7
	149	18.7	1.641	0.125	115 ± 13
10	140	18.1	1.880	0.129	112 ± 11
	153	15.4	0.935	0.101	100 ± 7
15	145	9.4	0.234	0.065	105 ± 4
	144	17.0	1.471	0.118	102 ± 7
20	147	16.7	1.040	0.114	105 ± 6

TABLE 3. ASSAY OF ESTRONE (OVIDUCT—16TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{640\ \mu\text{g.}}{160\ \mu\text{g.}}$$

N	b	s	t	λ	Pot. ratio ±S.E. %	Error range P = 0.05%
5	20.3	6.6	0.541	0.325	98 ± 33	−49; +95
	25.5	5.1	0.013	0.198	105 ± 21	−33; +51
	22.3	7.0	1.149	0.312	118 ± 38	−47; +90
7	20.6	6.2	0.457	0.302	101 ± 26	−41; +69
	23.7	5.0	2.810	0.211	113 ± 21	−31; +44
10	23.8	4.6	0.420	0.203	102 ± 15	−26; +34
	24.0	4.4	2.135	0.082	111 ± 15	−23; +31
15	22.7	5.2	1.069	0.228	107 ± 14	−24; +31

The tables list the values of N, b, s, t, λ, and the potency ratio ±S.E. N represents the number of sets at each dosage level, b the slope, s the standard deviation, t the significance of difference of the slopes, λ the ratio of s to b, and finally the potency ratio in terms of

the standard error of the potency ratio in percentage of original units employed for the potency ratio. To ascertain the total number of animals employed one multiplies the value N by four since each set contains 2 values on the unknown and 2 values on the standard.

The most favorable conditions for assay were found at the dosage levels of 640 μg. and 1280 μg. of estrone for the 9th day tests. Here the highest accuracy was obtained primarily due to the high value of the slope, b (table 2). In the 16th day tests, the dose levels of 1280 μg. and 640 μg. respectively showed the lowest error in the determination of the potency ratio. Actually the use of a total of 20 animals at 1280 μg. and 640 μg. will give a satisfactory assay using either the younger or older chicks. The younger animals showed a lower error in the potency ratio.

Of some 26 comparisons at the 9th day no instance of a significant difference in the slopes was found. Neither was a significant difference found in the slopes in any of the comparisons of the 16 day-old animals.

The Assay of Stilbestrol

All the animals were autopsied on the 9th day of life. The entire range of dosage levels from as little as 25 μg. total up to 1600 μg. yielded reasonably low error estimates with the lower doses having the higher reliability. Thus, using the range of 25 μg. and 50 μg. and a total of 20 animals a standard error of as little as ±10 per cent could be expected in the potency ratio (table 4). At the same dosage level an assay employing a total of 48 animals indicated a standard error in the potency ratio of ±7 per cent. In one instance of some 56 trials a significant difference in the slopes of the unknown and standard was found.

TABLE 4. ASSAY OF STILBESTROL (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{50\ \mu g.}{25\ \mu g.}$$

N	b	s	λ	t	Pot. Ratio ±S.E. %
5	98 91	6.46 11.7	0.066 0.129	0.413 1.900	98± 6 96±13
7	90 84	6.67 11.4	0.089 0.137	0.368 2.170	90±17 99±12
12	90	9.92	0.110	1.252	100± 7

The Assay of Estradiol Benzoate

Table 5 illustrates the assay of estradiol benzoate. Only one set of concentrations is represented, that is, 100 μg. and 200 μg. Although the slopes varied considerably from 52 to 193 the values for λ were relatively constant. The variation in λ was from 0.082 to 0.116. The

errors of the potency ratio were quite reasonable. Thus using a total of 20 animals a mean standard error of the potency ratio (mean = 103) was only ±11 per cent. With the use of twice the number of animals in one trial run a potency ratio of 103 per cent was found with a standard error of ±7 per cent. No significant differences were found in the slopes of the unknown and standard.

TABLE 5. ASSAY OF ESTRADIOL BENZOATE (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{200\ \mu g.}{100\ \mu g.}$$

N	b	s	λ	t	Pot. Ratio ±S.E. %
5	72	7.2	0.100	0.589	100 ± 10
	52	7.7	0.148	0.349	107 ± 16
	167	13.7	0.082	1.755	101 ± 16
	193	22.4	0.116	0.518	105 ± 13
7	66	7.0	0.106	0.217	103 ± 9
10	180	17.7	0.098	1.429	103 ± 7

The Assay of Estradiol

Representative data on the estradiol assay are tabulated in Table 6. The range of 400 μg. to 1600 μg. total dose of estradiol was studied and a reasonably accurate determination of the potency ratio was found. The best precision was found between the levels of 400 μg. and 800 μg. of estradiol. When a total of 40 chicks were used at this dosage level the potency ratio of 77 ± 11 per cent was found in one trial and 101 ± 21 per cent in a second trial. The known potency ratio was 100 per cent. In one trial using a total of 48 chicks the value was 80 ± 11 per cent.

TABLE 6. ASSAY OF ESTRADIOL (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{800\ \mu g.}{400\ \mu g.}$$

N	b	s	λ	t	Pot. Ratio ±S.E. %
5	101	21.3	0.211	0.678	72 ± 17
	147	29.2	0.200	0.092	87 ± 18
	62	26.6	0.430	1.072	112 ± 54
	65.7	10.0	0.150	1.408	92 ± 14
7	86.5	20.4	0.236	1.245	72 ± 15
	121	26.4	0.218	0.586	84 ± 16
	65.2	21.3	0.327	1.242	103 ± 29
	69	10.0	0.145	1.260	85 ± 11
10	121	23.4	0.193	0.548	77 ± 11
	64	18.4	0.288	1.643	101 ± 21
12	111	24.1	0.216	0.873	80 ± 11

DISCUSSION

The data pertaining to the use of the oviduct as a method of assay of estrogen are presented in condensed form in Table 7. The value N is similar to that previously used in the paper, that is, N represents

TABLE 7. COMPARATIVE MEAN STANDARD ERRORS IN PERCENT OF DETERMINATION OF POTENCY RATIO

(Numbers in parenthesis are number of trials)

Estrogen	Dose levels μg	Values of N					
		5	7	10	12	15	20
Stilbestrol	50/25	±10 (2)	±10 (2)	± 7 (1)			
	200/100	±16 (10)	±15 (2)	±11 (5)		± 9 (2)	
	400/100	±20 (5)		±16 (3)		±13 (1)	
	400/200	±20 (5)	±15 (2)	±13 (2)		±11 (1)	
	800/400	±26 (2)	±21 (2)	±17 (N=9) (1)			
	1600/400	±31 (2)	±27 (2)	±20 (N=9) (1)			
	1600/800		±45 (2)	±14 (N=9) (1)			
Estrone (9th day)	640/160	±30 (4)	±22 (3)	±19 (2)		±12 (1)	±15 (1)
	1200/160			±20 (2)		±16 (1)	±14 (1)
	1280/640	±13 (4)	±10 (2)	± 9 (2)		± 5 (2)	± 6 (2)
Estrone (16th day)	640/160	±29 (3)	±22 (2)	±14 (2)		±13 (1)	
	1280/640	±14 (3)	±16 (2)	±14 (2)		±10 (1)	
Estradiol Benzoate	200/100	±11 (4)	± 9 (1)	± 7 (1)			
Estradiol	800/400	±29 (4)	±21 (4)	±16 (2)	±14 (1)		
	1600/400	±35 (2)	±39 (2)	±28 (1)			
	1600/800	±26 (2)	±25 (2)	±20 (1)			

the number of groups of four animals each. Thus the total number of animals is equal to N times four.

Although stilbestrol assays may be done at dosage levels of 25 μg. to 1600 μg. per animal the best accuracy was realized in the region of 25 μg. to 200 μg. The relative shallowness of the log dose-response curve of the latter region is more than made up by the low order of variability at any given dose level. The assay is quite remarkable when one considers that the use of only a total of 20 animals at the 25 μg. and 50 μg. levels could yield a determination of the potency ratio with a standard error of only ±10 per cent.

The bioassay of estrone can be accomplished with good precision by this method but unfortunately relatively large amounts of material are needed. This seriously limits the value of the assay. With a total of 20 pullet chicks at the concentrations of 1280 μg. and 640 μg. mean standard errors of the potency ratio of ±13 per cent and ±14 per cent respectively were found for the chicks autopsied on the 9th and 16th day. The best accuracy for estrone assay was found at the combination of the 1280 and 640 microgram levels using a total of 60 chicks. Under these conditions a standard error of the potency ratio of only ±5 per cent was found.

Estradiol benzoate could be assayed at relatively low concentrations with high precision. With the combination of 200 and 100 microgram levels of this estrogen ester using a total of 20 pullet chicks there has been realized a mean standard error in potency ratio of only ±11 per cent. Keeping all conditions constant but increasing the total number of animals to 40 resulted in a lowering of the standard error to ±7 per cent.

The choice of breed of pullet chick for the oviduct assay is presented in another communication (Dorfman, 1947). The question of diet should receive attention since it has been shown that at least one vitamin is essential for the responsiveness of the oviduct. The work of Hertz (1945) which has been confirmed in this laboratory indicates that in the absence of folic acid the chick oviduct shows only a limited stimulation even in the presence of adequate concentrations of estrogens.

SUMMARY

A new bioassay procedure for estrogens has been presented which although not possessing the advantages of sensitivity does have a high precision. The method consists in treating pullets with estrogens for five consecutive days during the first 15 days of life and determining the oviduct response 24 hours after the last injection.

Applications of the new assay procedure have been described with respect to stilbestrol, estrone, estradiol, and estradiol benzoate. The responses of the oviduct to MDDA (methoxy bisdehydro doisynolic acid) have also been described. The responses of the oviduct to MDDA

are unique since relatively small amounts produce good responses but the log dose responses curve soon flattens out in spite of increased dosage.

At concentration levels of 25 μg. and 50 μg. of stilbestrol using a simplified statistical design, the potency ratio can be determined with standard errors of ±10 per cent and ±7 per cent respectively when totals of 20 and 40 pullet chicks were employed. Similar high accuracy was obtained for certain concentrations of estrone and estradiol benzoate.

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STUDIES ON THE BIOASSAY OF HORMONES— THE ASSAY OF PREGNANT MARE'S SERUM CHORIONIC GONADOTROPHIN

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THIS COMMUNICATION is concerned with studies on the bioassay of pregnant mare's serum chorionic gonadotrophin using the baby chick. Three endpoints have been studied. The oviduct response was found to be entirely unsuitable and the comb response of the male of doubtful value. However, the testis response appears to be a satisfactory endpoint having the advantages of reasonable sensitivity and reproducibility.

MATERIALS, ANIMALS, METHODS

The pregnant mare's serum hormone consisted of material kindly supplied by the Upjohn Company and the Schering Corporation. The material was readily soluble in water and was dissolved just before the first injections were done and kept at $-5°$ C. between injections. The total dose per animal was contained in 0.5 cc. of water.

The White Leghorn chicks were obtained from the Kerr Chickeries in Frenchtown, N. J., and usually arrived at the laboratory one day after hatching and immediately placed on a diet of starting mash and water.

For the oviduct studies pullet chicks were injected daily for 5 consecutive days starting with the fourth day of life and the injections continued through the eighth day. Twenty-four hours after the last injection the animals were killed with chloroform and the oviducts removed, blotted on a towel and weighed to the nearest 0.5 milligram on a torsion balance. The response was expressed as 100 times the ratio of the weight of the oviduct in milligrams to the body in grams.

The male chicks were studied in an analogous manner except that the combs and testis were removed at autopsy. The response of these organs also were expressed as 100 times the weight of the gland in milligrams to body weight in grams. One series of animals were used for the testis response to pregnant mare's serum hormone which were autopsied at 17 days of age. These animals received 5 injections of the hormone, one each day, from the twelfth through the sixteenth day of life. Autopsy was performed twenty-four hours after the last injection.

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Statistical Calculations

The experimental design was similar to that described by Bliss (1944) for the assay of penicillin. By this method is was possible to calculate the potency ratio, the error of the potency ratio and finally the significance of difference of the slopes. The various symbols used are similar to those employed by Bliss (1944). Thus N represents the number of sets used. Each set represented 2 animals on the standard and 2 animals on the unknown. Thus the total number of animals is equal to four times N. The slope is designated as b, s represents the standard deviation, and t represents Fisher's t value, a measure for the significancy of the differences between slopes of standard and unknown.

Essentially the experimental design was such that the following conditions were established:

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}.$$

The relationship tested was the logarithm of the dose versus the response. The exact formulations are not presented here but have been described by Bliss (1944).

EXPERIMENTAL

Oviduct Response

Although a significant increment in the ratio of the oviduct was found with 50 I.U. of pregnant mare's serum hormone, quadrupling the dose to 200 I.U. caused no further increase in the ratio. The increase observed was of a low order, in the range of 28 per cent. On the basis of these poor responses no attempt was made to evaluate the method quantitatively.

Comb Response

The comb response was studied in male and female chicks. In male chicks about 40 I.U. of pregnant mare's serum hormone was required to produce a significant increment in the comb ratio. Be-

TABLE 1. THE ASSAY OF PREGNANT MARE'S SERUM HORMONE BY MEANS OF THE COMB RESPONSE OF THE MALE CHICK

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

Unknown = Standard

(Chicks were 9 days of age at autopsy)

N	PMS conc. I.U.	b	s	λ	t	Potency ratio % ±S.E.	Error range P=0.95
8	40; 62.5	84.0	21.5	0.256	0.493	81+18	−36; +55
8	40; 125	62.8	22.8	0.364	0.975	165±50	−45; +83
8	62.5; 125	49.6	24.6	0.496	1.337	111±45	−56; +126

tween the levels of 40 to 125 I.U. a straight line relationship was found when the logarithm of the dose and response was considered. The equation for this relationship was $y = 50.4x - 36.5$. In the female chicks as large a dose as 200 I.U. caused no real increment in the comb ratio.

Table 1 illustrates the use of the comb response as a method for the assay of the pregnant mare's serum hormone. Using 8 sets of animals or a total of 32 animals resulted in errors in the determination of the potency ratio ranging from −36 per cent and +55 per cent to −56 per cent and +126 per cent at $P = 0.95$.

Testis Response

A straight line realationship has been shown to exist between

TABLE 2. THE ASSAY OF PREGNANT MARE'S SERUM HORMONE USING THE TESTIS RESPONSE OF THE CHICK

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

Standard = Unknown

(Chicks were 9 days of age at autopsy)

N	Conc. PMS I.U.	b	s	λ	t	Potency ratio % ±S.E.	Error range P = 0.95
14	25; 75	48.5	16.0	0.331	1.051	91 ±19	−34; + 50
8	25; 75	57.5 47.7	15.5 18.0	0.270 0.377	1.172 0.843	88 ±19 84 ±26	−36; + 55 −46; + 85
8	62.5; 125	115	30.6	0.266	1.001	94 ±20	−35; + 55
8	62.5; 250	66.7	23.9	0.354	0.280	143 ±42	−45; + 81
8	125; 500	58.2	36.5	0.625	0.990	96 ±49	−64; +177
8	250; 500	98.3	27.0	0.275	0.053	133 ±32	−38; + 62
8	62.5; 500	77.5	18.4	0.238	0.286	134 ±26	−32; + 48

the logarithm of the dose and the testis response. The equation for the logarithm of the dose response relationship was found to be $y = 87.2x - 94.9$.

From Table 2 it is seen that an accuracy of −34 per cent to +50 per cent can be realized in the determination of the potency ratio when a total of 56 animals are used. This was not much better than when only 32 animals were employed. In the dose range of 25 to 125 I.U. the error range was somewhat better than in the range 125 to 500 I.U.

One series of chicks were studied in which the hormone was administered once daily for 5 days starting with the twelfth day of life.

Autopsy was performed at 17 days of age. The control animals showed testis ratios considerably greater than was found for the animals autopsied at 9 days of age. Thus the younger animals showed a mean ratio of 18 ± 2 while the older animals showed a mean ratio of 31 ± 3. Further a striking difference was found in the relative sensitivities of the testis of the two age groups. As little as 25 I.U. of hormone caused a significant increment in testis mean ratio in animals autopsied at 9 days of age, an increase from 18 ± 2 to 37 ± 2. In the older animals the amount necessary to produce a significant increment in testis mean ratio was between 80 to 160 I.U. of the pregnant mare's serum hormone. The control mean ratio for the older animals was 31 ± 3 while 20 I.U. and 40 I.U. dosages produced mean ratios of 28 ± 1 and 30 ± 2 respectively. At 80 I.U. the mean ratio was 35 ± 2 and at 160 I.U. a mean ratio of 52 ± 3 was found. Using the Bliss (1944) design with the dosage levels of 80 I.U. and 160 I.U. a comparison was run comparing a standard and unknown where the standard was identical to the unknown. Using a total of 40 chicks a potency ratio of 113 per cent ± 20 per cent was found as compared to a theoretical 100 per cent. At $P = 0.95$ an error range of -30 per cent to $+42$ per cent was found.

CONCLUSIONS

The chick can serve as a suitable assay animal for pregnant mare's serum hormone if the testis response is employed. The comb size of the male has a more limited usefulness but the comb stimulation in the female was of no value under the conditions studied. The oviduct response was of no value. Thus the fact that only the smallest sort of stimulation of the oviduct could be demonstrated is not surprising since the relatively large amounts of estrogen that are required for oviduct stimulation (Dorfman, 1947). Thus about 40 micrograms of estrone and about 20 micrograms of estradiol are required to produce the stimulation found for 50 I.U. of pregnant mare's serum over the same period of time (5 days).

Of interest is the simultaneous increase in weight of the testis and the comb when stimulated with varying amounts of pregnant mare's serum hormone. Thus a straight line function for the testis growth was found from 25 to 500 I.U. without signs of the curve falling off. However, the comb growth increments seems to fall off beyond 62.5 to 125 I.U. This appears to indicate that certain nonandrogen producing elements of the testis continue to be stimulated. Or in other words it may be said that the androgen producing elements of the testis are quickly stimulated maximally.

A number of methods have been employed for the assay of pregnant mare's serum hormone. These consist of follicle stimulation, ovulation, corpus luteum formation, and estrus reactions in the vagina of the immature rat. No systematic statistical analysis for errors of

determination have been presented. Cartland and Nelson (1937) have used the ovarian weight of the immature rat as the endpoint for assay of the pregnant mare's serum hormone. The rats were Wistar strain animals weighing between 30 and 40 grams at 21 and 23 days of age. The hormone was administered in 3 daily doses and autopsy was performed 24 hours after the last injection. The aim of the assay was to produce mean pairs of ovaries equal to 65 mg. which was approximately 5 times the size of the ovaries of the normal untreated animals. By the use of 20 to 40 animals the authors claim that "this method has yielded reproducible results within an experimental error of ± 10 per cent." No supporting data accompany this report.

SUMMARY

Pregnant mare's serum hormone can be assayed by means of the response of the chick's testis. Using a total of 32 animals (16 on the standard and 16 on the unknown) and using the experimental design of Bliss (1944) the potency ratio can be assessed with an error range of about -34 per cent to $+50$ per cent at $P = 0.95$.

The response of the chick's oviduct to pregnant mare's serum has been found unsatisfactory as an assay procedure. The use of the male chick's comb, but not the female chick's comb, is satisfactory for quantitative assay work. The comb test is inferior, however, to the testis test.

A much greater change in testis weight can be produced with increasing dosage of pregnant mare's serum hormone than can be produced in the comb. This seems to indicate that certain nonandrogen producing elements of the testis continue to be stimulated after the maximal response of the androgen producing elements have been reached.

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STUDIES ON THE BIOASSAY OF HORMONES—THE COMPARATIVE ACTIVITY OF ESTROGENS BY THE OVIDUCT RESPONSE IN CHICKS

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A METHOD employing the chick oviduct response previously described (Dorfman, 1947) has been employed to study the comparative activities of estrone, estradiol, stilbestrol, MDDA (methoxy bisdehydro doisynolic acid), and estradiol benzoate. As a result of an extended study of the increasing response with increasing dose of these compounds it was possible to pick regions of the respective log dose-response curves where the responses gave parallel lines for the evaluation of the comparative activities of various estrogens.

ANIMALS, METHOD AND MATERIALS

All chicks were White Leghorns and were supplied by Kerr Chickeries, Inc., Frenchtown, New Jersey. During the course of the investigations they were fed chick starting mash and water.

The standard procedure consisted of dissolving the test material in corn oil so that the total dose was contained in 0.5 cc. of oil. The material was injected subcutaneously once daily for 5 days (0.1 cc. of solution per day) from the 4th day of life through the 8th day of life. Twenty-four hours after the last injection the animals were sacrificed with chloroform and the body weights and oviduct weights were determined. All results are considered in terms of 100 times the ratio of the oviduct weight in milligrams to the body weight in grams.

The estrone, estradiol, estradiol benzoate, and MDDA were supplied by Ciba Pharmaceutical Products, Inc., and the stilbestrol was supplied by Wintrop Chemical Co., Inc.

The statistical calculations were patterned after Fischer (1934), Irwin (1937), and Bülbring (1935).

RESULTS

Table 1 lists the dosage levels studied and used for the calculations of the potency ratio, and the error range of the potency ratio.

Stilbestrol, MDDA, estradiol, and estradiol benzoate were found to have slopes which were not significantly different by the method

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of Fischer (1934). In the case of estrone, the slope varied significantly from stilbestrol but not from that of estradiol benzoate. The relative potency of estrone in terms of stilbestrol was determined both directly and indirectly compared to estradiol benzoate. These considerations are listed in Table 2.

TABLE 1. RESPONSE OF CHICK'S OVIDUCT TO ESTROGENS

Compound	Dosage level μg.	Number of chicks	Mean oviduct ratio[1]	Standard deviation of mean
Stilbestrol	50	24	55	11
	100	54	71	17
	200	53	143	47
MDDA	100	18	17	12
	400	42	101	181
Estradiol	400	52	41	10
	800	21	69	19
	1600	23	105	34
Estradiol Benzoate	100	44	22	7
	200	42	59	25
	400	23	123	41
Estrone	640	68	31	9
	1280	40	75	7

[1] The ratio is 100 times the weight of the oviduct in milligram divided by the body weight in grams.

TABLE 2. THE RELATIVE POTENCIES OF VARIOUS ESTROGENS ON THE CHICK'S OVIDUCT

(Stilbestrol = 100 per cent)

Compound	Relative potency %	Error range % (P = 0.95)
Stilbestrol	100	—
MDDA	42	−29; +41
Estradiol	18	− 9; + 9
Estradiol Benzoate	48	−10; +10
Estrone	12	− 7; + 7
	(12)*	(−5; + 5)

* This was found by comparing estrone to estradiol benzoate which was compared to stilbestrol in turn.

A further word may be mentioned about the activity of the estrogen MDDA. It will be noted that the standard deviation of the mean at both the 100 and 400 μg. levels is large as compared to that found for the other estrogens. This is due to the unusually wide dispersion of the individual observations.

DISCUSSION

This method affords a convenient and relatively accurate method for the studies on the comparative activity of estrogens. The fact that MDDA is assessed as 42 per cent of the activity of stilbestrol is a bit misleading. This relative activity only holds in the lower dosage ranges since further increases in dosage of stilbestrol results in great increases in the response of the oviduct. This is not quite true for MDDA since only a fraction of the animals show sizable responses at the higher doses.

Estradiol was found to be only 18 per cent as active as stilbestrol which is a rather low comparative activity. The increased activity of the estradiol benzoate is not unexpected since the standard method of administration consisted of one injection per day. The benzoate is well known as a derivative having a prolonged action.

Estrone has quite a low activity but of the same order as estradiol. Thus estrone was found to be 67 per cent as active as estradiol and only 12 per cent as active as stilbestrol.

Monroe and Kosin (1940; 1946) have used the chick oviduct method for the evaluation of the relative potencies of both naturally occurring estrogens and certain synthetic estrogens. The studies on the relative potencies of synthetic estrogens were done by incorporating the estrogen in the commercial chick starting mash at a level of 30 mg. of estrogen per pound of feed. By this method they found the following descending order of potency of six compounds as follows: dianisylhexane (dimethyl ether of hexestrol), dianisylhexene (dimethyl ether of stilbestrol), hexestrol, dienestrol, dianisylhexadiene (dimethyl ether of dienestrol), and stilbestrol. The studies of Munroe and Kosin (1940) on the naturally occurring estrogens using the chick oviduct response established the following descending order of activity: estradiol dipropionate, estradiol benzoate, estradiol, and estrone. No attempt was made, however, to quantitate the relative activities of the various estrogens studied.

SUMMARY

The comparative activities of four estrogens were studied using the oviduct response of the week-old White Leghorn chick. Using stilbestrol as the reference standard expressed as 100 per cent, MDDA (methoxy bisdehydro doisynolic acid) had a potency of 42 per cent; estradiol, 18 per cent; estradiol benzoate, 48 per cent; and estrone a relative potency of 12 per cent. The error range of the potency ratio of MDDA was found to be -29 to $+41$ per cent at $P = 0.95$ due to the high variation in the responses of the chick's oviducts to this estrogen. The other three relative potencies were determined with an accuracy of ± 10 per cent or better.

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STUDIES ON THE BIOASSAY OF HORMONES— THE COMPARATIVE OVIDUCT RESPONSE OF VARIOUS BREEDS OF CHICKS TO STILBESTROL

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IN PREVIOUS COMMUNICATIONS we have reported the use of the chick oviduct for the quantitative assay of such estrogens as stilbestrol, estrone, estradiol, estradiol benzoate and methoxy bisdehydro doisynolic acid (MDDA) (Dorfman, 1947A). This method was also employed to study the relative potencies of estrone, estradiol, estradiol benzoate, MDDA, and stilbestrol (Dorfman, 1947B). The question arises as to the relative sensitivity of various breeds of chicks to oviduct stimulation by estrogens. It was also of interest to determine the relative variation among individuals of the various breeds to the view of finding the most accurate conditions for the bioassay of estrogens.

ANIMALS, METHODS, AND MATERIAL

Four breeds of chicks, White Leghorns, White Rocks, Rhode Island Reds and a Sex Linked Cross (Plymouth Rock and New Hampshire Red) were obtained from Kerr Chickeries in Frenchtown, N. J.

The procedure consisted of dissolving the stilbestrol,[1] the estrogen chosen for all of these studies, in corn oil so that the total dose per animal was contained in 0.5 cc. of oil. The material was injected subcutaneously once daily for 5 days (0.1 cc. of oil solution per day) from the fourth day of life through the eighth day of life. Twenty-four hours after the last injection the animals were sacrificed with chloroform and the body weights and oviduct weights were determined. All results are considered in terms of 100 times the ratio of the oviduct weight in milligrams to the body weight in grams.

The calculations of the relative potencies (M), the standard error ($S.E.$) of the potency ratio, and the significancy of difference between slopes of the unknown and standard (t) were done by the method of Bliss (1944). The statistical calculations involving the relative sensitivities of the various breeds of chicks to estrogens were done by the methods described by Fischer (1934), Irwin (1937) and Bülbring (1935).

In the studies involving the determination of relative potencies the theo-

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[1] The stilbestrol was kindly supplied by the Winthrop Chemical Company, Inc.

retical potency ratio was always 100 per cent since the unknown and standard were identical.

EXPERIMENTAL

Table 1 illustrates the oviduct response of the four breeds of chicks to stilbestrol between the ranges of 50 μg. and 400 μg. of estrogen.

TABLE 1. OVIDUCT RESPONSES OF FOUR BREEDS OF CHICKS TO STILBESTROL

Breed	Total amount of stilbestrol administered μg.	Number of chicks	Oviduct Ratio ±S.E.
White Leghorn	0	21	11 ± 1
	50	24	55 ± 2
	100	54	71 ± 2
	200	53	143 ± 6
	400	74	270 ±10
White Rock	0	12	20 ± 2
	50	21	57 ± 1
	100	22	86 ± 5
	200	20	156 ±10
	400	22	239 ±20
Rhode Island Red	0	10	17 ± 1
	50	19	47 ± 3
	100	20	84 ± 5
	200	20	174 ±11
	400	17	290 ±38
Sex Linked Cross (Plymouth Rock and New Hampshire Red)	0	12	17 ± 2
	50	23	43 ± 2
	100	21	103 ± 8
	200	22	209 ±13
	400	20	284 ±16

TABLE 2. COMPARATIVE REPRODUCIBILITY OF THE OVIDUCT ASSAY FOR STILBESTROL WHEN DIFFERENT BREEDS OF CHICKS WERE USED

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{200\ \mu g.}{100\ \mu g.}$$

(A total of 40 chicks were used for each comparison)

Breed	b	s	λ	t	M ±S.E.	Error range % (P=0.95)
White Leghorn	391	56.9	0.145	0.322	105 ±11	−19; +23
	334	72.4	0.216	0.183	88 ±14	−27; +37
	300	41.5	0.138	0.995	93 ± 9	−18; +21
	205	22.8	0.111	0.246	105 ±12	−20; +26
	204	21.2	0.104	0.226	100 ± 7	−14; +15
White Rock	233	37.3	0.160	1.031	83 ±10	−21; +26
Rhode Island Red	596	33.0	0.055	2.341	112 ± 5	− 8; + 9
Sex Linked Cross	358	57.3	0.160	1.110	97 ±11	−21; +26

The comparative reproducibility of the oviduct assay for stilbestrol using four different breeds of chicks is represented in Table 2. The concentrations of stilbestrol are 100 μg. and 200 μg. and the calculations are by the method of Bliss (1944). In each case the total number of animals used was 40 (20 chicks on the standard and 20 chicks on the unknown). The mean error range at $P=0.95$ for the Rhode Island Reds are less than for the other three breeds.

Tables 3 and 4 further illustrate the comparative reproducibility of the oviduct assay for stilbestrol at 100 μg. and 400 μg. and 200 μg. and 400 μg. respectively. The error ranges found for the various breeds at 100 μg. and 400 μg. are quite similar. The White Leghorns however, show a smaller error range at concentrations of 200 μg. and 400 μg.

TABLE 3. COMPARATIVE REPRODUCIBILITY OF THE OVIDUCT ASSAY FOR STILBESTROL WHEN DIFFERENT BREEDS OF CHICKS WERE USED

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{400\ \mu\text{g.}}{100\ \mu\text{g.}}$$

(A total of 40 chicks were used for each comparison)

Breed	b	s	λ	t	M $\pm$S.E.	Error range % (P=0.95)
White Leghorn	306	60.6	0.198	0.234	108 $\pm$16	−26; +35
	304	77.6	0.255	1.480	85 $\pm$16	−32; +46
	318	59.1	0.186	0.305	96 $\pm$13	−24; +31
White Rock	256	76.5	0.300	0.875	113 $\pm$25	−35; +55
Rhode Island Red	324	13.4	0.414	0.171	88 $\pm$27	+45; +82
Sex Linked Cross	306	97.5	0.316	0.761	70 $\pm$16	−37; +59

TABLE 4. COMPARATIVE REPRODUCIBILITY OF THE OVIDUCT ASSAY FOR STILBESTROL WHEN DIFFERENT BREEDS OF CHICKS WERE USED

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{400\ \mu\text{g.}}{200\ \mu\text{g.}}$$

(A total of 40 chicks were used for each comparison)

Breed	b	s	λ	t	M $\pm$S.E.	Error range % (P=0.95)
White Leghorn	391	56.9	0.145	0.322	105 $\pm$11	− 9; +11
	334	72.4	0.216	0.183	88 $\pm$14	−27; +37
White Rock	278	81.0	0.291	1.311	100 $\pm$21	−34; +53
Rhode Island Red	351	11.9	0.331	0.840	106 $\pm$24	−38; +62
Sex Linked Cross	253	98.5	0.389	1.390	78 $\pm$21	−43; +76

Comparative Oviduct Sensitivity of Various Breeds to Stilbestrol

Tables 5 and 6 illustrate the comparative sensitivity of the various breeds to oviduct stimulation by stilbestrol. Table 5 lists the regression coefficients calculated for the four breeds from the responses at 100, 200, and 400 g. of stilbestrol. From these data it was possible to quantitate the relative sensitivities which are presented in Table 6. The Sex Linked Crosses and the Rhode Island Reds appear to be the more sensitive. However, the White Leghorn and White Rocks have a sensitivity of about 77 per cent of the other two breeds.

TABLE 5. REGRESSION COEFFICIENTS CALCULATED FOR FOUR BREEDS OF CHICKS AT DOSAGES OF 100, 200, 400 G. STILBESTROL

Breed	Total number of animals	$\bar{x}$	Slope b	Constant a
White Leghorn	181	2.333	339	173
White Rock	64	2.301	255	167
Rhode Island Red	57	2.256	422	176
Sex Linked Cross	63	2.295	293	197

TABLE 6. THE COMPARATIVE SENSITIVITY OF VARIOUS BREEDS OF CHICKS TO STILBESTROL

Breed	Total number of chicks	Relative sensitivity %	Error range P=0.95 %
Sex Linked Cross	63	100	—
Rhode Island Red	57	94	−15; +17
White Leghorn	181	77	−11; +13
White Rock	64	77	− 7; + 8

DISCUSSION AND CONCLUSION

These experiments were designed to answer two questions: A) What is the relative sensitivity of oviducts of various species to estrogens; and B) Is there any significant difference between the various breeds of chicks from the standpoint of overall accuracy for bioassay?

No great difference in oviduct sensitivity to stilbestrol was found for the four breeds studied. Actually the Sex Linked Crosses and Rhode Island Reds were the most reactive and no significant difference between the two breeds. The White Leghorn and White Rock breeds showed a significantly lower sensitivity. These breeds were found to possess 77 per cent of the reactivity of the Sex Linked Crosses.

From the standpoint of overall accuracy for bioassay there ap-

peared to be an advantage in using the White Leghorn breed. In a direct comparison between breeds the White Rocks, Rhode Island Reds, and the Sex Linked chicks gave a mean error range at P = 0.95 of −30 to +43 per cent; −30 to +43 per cent; and −33 to +52 per cent respectively, for a total of 40 chicks. Under similar conditions the White Leghorns gave a mean error range of −21 to +28 per cent.

SUMMARY

Four breeds of chicks, White Leghorns, White Rocks, Rhode Island Reds, and a Sexed Linked breed were studied with respect to oviduct sensitivity to stilbestrol. Rhode Island Reds and the Sexed Linked breed were the more sensitive. The other two breeds, the White Leghorns and White Rocks showed an oviduct sensitivity of 77 per cent of the Sex Linked chicks.

For use in bioassay the White Leghorn appeared to be the best from the standpoint of the lowest error range. Using a total of 40 chicks (20 on the unknown and 20 on the standard) the White Rocks, Rhode Island Reds, Sex Linked Cross, gave a mean error range at P = 0.95 of −30 to +43 per cent; −30 to +43 per cent; and −33 to +52 per cent respectively while under the same conditions the White Leghorns gave a mean error range of −21 to +28 per cent.

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THE ACCUMULATION OF RADIOACTIVE IODIDE BY THE THYROID GLAND IN NORMAL AND THYROTOXIC SUBJECTS AND THE EFFECT OF THIOCYANATE ON ITS DISCHARGE[1]

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INTEREST in the effects of thiocyanate on the thyroid gland followed the observation that goiter occurred in 11 of 246 patients treated with the material for long periods of time for hypertension (Barker, 1936; Barker, Lindberg, and Wald, 1941). Both the thyroid enlargement and the accompanying manifestations of hypothyroidism were relieved by thyroid administration. It was later postulated that the action of thiocyanate in producing goiter in rats differed from that of the thiouracil derivatives (Astwood, 1943). With the former the thyroid effect was much slower to develop and was completely abolished by added iodide, which did not modify the thyroid response to compounds of the thiouracil series. From studies of the accumulation of I^{131} by surviving thyroid slices from sheep, Franklin, Chaikoff, and Lerner (1944) showed that the iodide-concentrating capacity was inhibited by thiocyanate. Other goitrogens did not significantly diminish the accumulation of iodide by the tissue but prevented its conversion to diiodotyrosine and thyroxin. In intact animals, the administration of antithyroid substances caused the thyroid to enlarge and its iodide concentration to diminish (Astwood and Bissell, 1944). In such animals, however, the quantity of iodine remaining in the thyroid after treatment with thiouracil was found to be related to the iodine intake. If the iodine intake were greatly increased, large concentrations of iodine remained in the gland, but this iodine was shown not to be protein bound (McGinty and Sharp, 1946). Furthermore, the thyroids of rats depleted by thiouracil could entrap large quantities of injected iodide (Astwood, 1944–45). In thiouracil-treated animals, the entrapped iodide was spontaneously discharged so that about one-half was lost in five to six hours and a considerable portion of the remainder during the next eighteen hours (VanderLaan and Bissell, 1946). The *in vitro* experiments of Franklin, Chaikoff, and Lerner were confirmed by Wolfe, Chaikoff, Taurog,

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and Rubin (1946), who showed that thiocyanate inhibited the accumulation of radioactive iodine by the thyroid gland of rats. Thiocyanate also strongly interfered with the accumulation of iodide by thyroids under the influence of propylthiouracil, provided the injected dose of iodine was small; with larger amounts, this inhibition was less striking (VanderLaan and Bissell, 1946). The iodine accumulated in the thyroid gland while under the influence of propylthiouracil was ultrafiltrable and behaved as iodide ion when studied potentiometrically using the silver-silver iodide electrode (VanderLaan and VanderLaan, 1947). These findings provided further evidence that the material was in the form of iodide. These same investigators found that thirty or more times the normal content of iodide was accumulated in thyroids made hyperplastic by propylthiouracil, even though the iodide concentration of the serum was not elevated. They further noted that thiocyanate not only prevented the uptake of iodide, but that it caused a rapid discharge of the iodide accumulated while the glands were under the influence of propylthiouracil.

The present report extends these studies by observations on normal human beings and subjects with hyperthyroidism without therapy and during the course of treatment with antithyroid drugs. It has been observed that a fully effective dose of an antithyroid compound given prior to the administration of a tracer dose of iodine profoundly modifies the course of iodine accumulation in the thyroid region. Under these circumstances radioactive material is rapidly concentrated in the thyroid gland, but while the total amount accumulated there is reduced, the maximal concentration is achieved in the brief interval of one to two hours. Thereafter the radioactive iodine slowly leaves the thyroid region; if the antithyroid treatment be maintained at a fully effective level, the iodine is completely lost from the gland in a few days. When the concentration of iodine in the thyroid is maximal, a large dose of potassium thiocyanate or of potassium iodide causes a rapid discharge of the radioactive iodine from the gland. The completeness of the disappearance of the previously accumulated radioactive iodine is related to the effectiveness of the antecedent antithyroid medication and to the dose of potassium thiocyanate or potassium iodide. Individuals suffering from hyperthyroidism exhibit a larger accumulation of iodide than normal persons, and the possibility of using this difference as a diagnostic method has therefore been tested. By using doses of potassium thiocyanate or potassium iodide which were found to be nearly completely effective, this phenomenon made it possible to study the effectiveness of the antithyroid pretreatment.

METHODS

Various amounts of different antithyroid drugs were ingested prior to the administration of the radioactive iodine in order to inhibit thyroid hor-

mone formation. In the studies designed to test the method as a means of diagnosis of hyperthyroidism, and in the several experimental procedures on normal subjects, it was desirable to attain as nearly complete inhibition of hormone formation as possible; this was accomplished conveniently by the oral administration of 100 milligrams of 2-mercaptoimidazole one-half to two hours before giving the I^{131}. When it was desired to estimate the degree of inhibition of hormone formation and, hence, the effectiveness of a therapeutic schedule in patients already receiving treatment, no additional antithyroid medication was given, the iodine being administered at the desired interval after the usual dose of drug. In all instances 100 microcuries of I^{131} without added carrier in weakly alkaline solution diluted to about 25 cc. with normal saline was given by mouth, followed by about 25 cc. of water to wash the material from the flask and the mouth and esophagus. Serial counts were then made at ten- to thirty-minute intervals over the isthmus of the thyroid gland with a shielded Geiger-Muller counter as described previously (Stanley and Astwood, 1947). When the accumulation of I^{131} in the thyroid region reached a maximum, as indicated by successive counts of the same magnitude, potassium thiocyanate, usually one gram, freshly dissolved in 25 to 50 cc. of water, was given by mouth. Counts were made frequently during the next hour to follow the discharge of I^{131} from the gland. The rate of loss was maximal during the first half hour after the ingestion of potassium thiocyanate and usually ceased during the second or third hour. The "background" was determined by counting over the left anterior chest at appropriate intervals during the procedure.

RESULTS

Normal Subjects: In 27 normal subjects after nearly complete inhibition of thyroid hormone formation by mercaptoimidazole the accumulation of iodide by the thyroid gland was slight, and the resulting curves quite unspectacular (Figures 1 and 2 and Table 1). The quantity of I^{131} accumulated was limited, and the maximum of less than 35 counts per second was reached in $1\frac{1}{2}$ to $2\frac{1}{2}$ hours. During the hour following potassium thiocyanate administration, the radioactive iodine was discharged from the thyroid at a rate similar to the rate of collection. The loss of I^{131} was frequently not quite complete at the end of the procedure, and counts were noted 2 to 4 counts per second higher over the thyroid than those of the general body background. Although these differences were quite small, they probably indicated significant concentrations of I^{131} in the thyroid gland, since after the administration of I^{131} to certain myxedematous patients, whose atrophic thyroids were incapable of accumulating the material, the radioactivity was found to be greater over the chest than over the thyroid region. On the other hand, in several of the normal subjects with very small uptakes of iodine, the radioactivity over the thyroid was less than background at the end of the test (Table 1) indicating that nearly complete discharge occurred under the influence of thiocyanate. When thyroid hormone formation was presumably completely suppressed by 100 milligrams of mercaptoimidazole and

the potassium thiocyanate was given *before* the I^{131}, there was little or no collection of the radioactive iodine by the thyroid gland as shown by the failure of the count over the thyroid to rise above that of the body background (No. 7, Table 1). In Figure 2 are shown the curves obtained after administration of I^{131} to two normal sub-

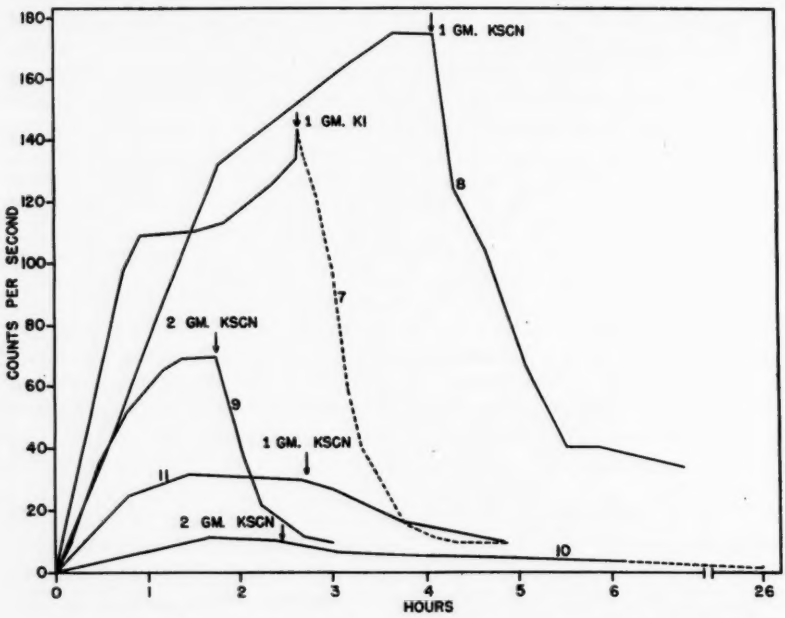

FIG. 1. The radio-iodide uptakes of two normal subjects (Nos. 10 and 11) and three patients with thyrotoxicosis (Nos. 7, 8, and 9). Each person had received 100 milligrams of mercaptoimidazole by mouth 65 to 80 minutes before administration of radioactive iodine to inhibit hormone formation. Nos. 8 and 11 took one gram and Nos. 9 and 10 two grams of potassium thiocyanate, while No. 7 was given one gram of potassium iodide, at the indicated times, also by mouth. Note the large iodide collection by the thyrotoxic patients as compared to the control subjects. When relatively complete inhibition was attained as in these individuals, the loss of the collected radio-iodide was more rapid than its accumulation in the thyrotoxic patients, while in the normals the rates of uptake and discharge were similar. The same data are given in Tables 1 and 2; the numbers are the same in the tables and in the above figure.

jects under the conditions described above, except that no potassium thiocyanate was given (see also Table 1). The rate of discharge of iodide was slow in all of the normal subjects; in those individuals with the smallest accumulation of I^{131} (maximal counts of 10 to 15 counts per second), there seemed to be little difference whether or not potassium thiocyanate was given, as the rate of discharge was similarly slow under both conditions. In three subjects no antithyroid agent was given before the radioactive iodine. The subsequent administra-

tion of 0.5 to 1.0 gram of potassium thiocyanate had little effect upon the course of iodine accumulation in the thyroid region and only a partial, transitory inhibition was observed. Doses of 10 milligrams or more of ordinary potassium iodide given after radioactive iodine merely caused the uptake of radioactive iodine by the thyroid to cease. Iodine which had already accumulated in the thyroid was not diluted out, indicating that it had quickly become bound to protein.

Thyrotoxic Patients: In each of 42 patients studied by a total of 90 tests (Figures 1, 2 and Table 2), the diagnosis of thyrotoxicosis was made on the basis of the usual clinical and laboratory grounds. No

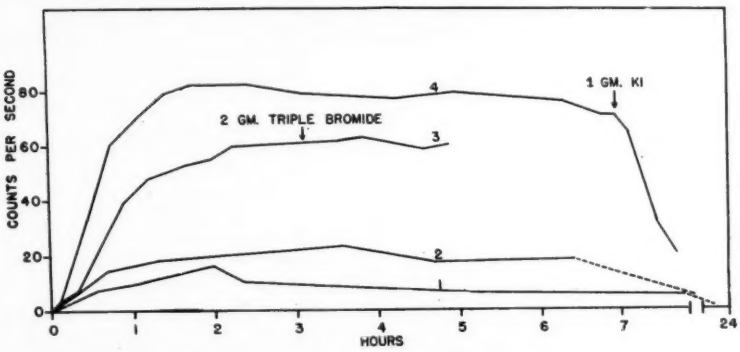

FIG. 2. The radio-iodide accumulation curves of two additional normal persons (Nos. 1 and 2), and two thyrotoxic patients (Nos. 3 and 4) under treatment with antithyroid drugs. All subjects had been given premedication to suppress thyroid hormone formation as indicated in Tables 1 and 2 (the numbers correspond to the same patients in the above figure and in Tables 1 and 2). These curves demonstrate the course of the radio-iodide levels in the thyroid gland when an inactive substance, such as the bromide ion (No. 3), was administered, or when no thiocyanate or ordinary iodide was given. It will be seen that the spontaneous loss of radioactive iodide was very gradual. In patient 4, one gram of ordinary potassium iodide produced the usual discharge seven hours after the tracer was taken. This indicated that the major portion of the I^{131} was still in the form of iodide ion nine hours after the last dose of 20 milligrams of mercapto-imidazole. Presumably organic binding of iodide was effectively inhibited for at least this interval by this dose of mercaptoimidazole.

therapy of any sort had been administered prior to the test in ten patients (5 through 14, Table 2), with the exception of the 100-milligram dose of mercaptoimidazole given in all instances before the procedure to inhibit thyroid hormone formation. The pattern of accumulation and discharge of radioactive iodine was quite distinctive. There was a rapid uptake by the thyroid gland during the first one to two hours, with the level of the maximum higher than 60 counts per second in all cases. The rate of the spontaneous loss of the collected iodide was about the same as in the normal subjects (Figure 2 and Table 2). Within a few minutes after the administration of potassium thiocyanate by mouth, the radioactivity in the thyroid

decreased in a spectacular fashion, and the rapid discharge of accumulated iodide was usually completed in less than two hours. In most instances the count over the thyroid at the end of the test failed to reach the level of the background; this difference (2 to 24 counts per second) was usually greater than that which occurred in normal subjects. Too, when thiocyanate was taken before the I^{131} by thyrotoxic

TABLE 1. THE QUANTITY OF RADIOACTIVE IODIDE ACCUMULATED IN THE THYROID REGION IN 20 NORMAL SUBJECTS FOLLOWING THE ADMINISTRATION OF 100 MICROCURIES OF I^{131}. ALL SUBJECTS WERE TREATED WITH 100 MILLIGRAMS OF MERCAPTOIMIDAZOLE ONE-HALF TO TWO HOURS BEFORE THE TEST. THE MAXIMAL VALUE, THE TIME WHEN THE MAXIMUM WAS REACHED, AND THE EFFECT OF A SINGLE DOSE OF POTASSIUM THIOCYANATE ARE SHOWN IN SUBJECTS 7–20. IN SUBJECTS 1–6, THE SPONTANEOUS COURSE AFTER THE MAXIMUM ACCUMULATION AND WITHOUT THIOCYANATE IS DEPICTED

	Maximum accumulation	Time of maximum after I^{131} administration	Dose of KSCN	Course of I^{131} loss (No Thiocyanate) Time in hours after maximum					Background**
				1 hr.	2 hr.	3 hr.	4 hr.	24 hr.	
	c/s	Hours	Grams	c/s	c/s	c/s	c/s	c/s	c/s
1.	17	2:00	0	10	8	7	6	2	5–7
2.	23	3:34	0	18	18	18	—	5	5–7
3.	27	1:06	0	27	26	26	—	6	—
4.	15	:54	0	11	11	9	8	4	4–7
5.	12	:50	0	10	9	9	8	—	—
6.	19	2:23	0	17	16	16	17	—	—
				Course after KSCN					
7.	7	:48	1*	6	6	—	—	—	6–8
8.	9	1:09	2	5	4	4	4	1	4
9.	10	1:06	1	5	5	—	—	—	6–7
10.	11	1:40	2	6	5	4	3	—	4–7
11.	32	1:27	1	16	9	—	—	—	7
12.	21	2:22	2	13	8	—	—	—	5
13.	27	1:28	1	10	—	—	—	2	1
14.	33	1:28	1	10	6	—	—	—	4–5
15.	18	1:13	1	6	—	—	—	—	3–6
16.	20	1:10	1	10	8	7	—	—	4
17.	30	:42	1	8	6	—	—	2	—
18.	20	:56	1	13	9 (1:36)	—	—	—	5–7
19.	25	:56	2	9	—	—	—	—	6
20.	25	1:47	1	10	—	—	—	—	7

* This patient received the potassium thiocyanate 18 minutes before the I^{131} was given.

** Obtained by counting over the upper anterior chest.

patients (Table 2, No. 1), the count over the thyroid was not prevented from rising to a level which was above the background count.

Determination of the Efficiency of Treatment with Antithyroid Compounds: The response to antithyroid medication in thyrotoxicosis depends upon such factors as the potency, the duration of action, the dosage and the individual sensitivity to the administered drug, previous iodine therapy, and the amount of hormone stored in the gland. Thus, evaluation of the efficacy of any compound is at best a lengthy process with many uncontrollable variables; at times correct judg-

ment of the efficacy of treatment by the usual clinical methods is impossible. The action of the thiouracil group of antithyroid drugs is a very selective one. These compounds cause an inhibition of the organic binding of iodine (which must first have been accumulated as iodide) to protein or in other words, these substances interfere

TABLE 2. THYROXOTIC PATIENTS: THE QUANTITY OF RADIOACTIVE IODIDE ACCUMULATED IN THE THYROID REGION IN PATIENTS WITH THYROTOXICOSIS WHO WERE GIVEN 100 MILLIGRAMS MERCAPTOIMIDAZOLE ONE TO TWO HOURS BEFORE THE TEST. THE EFFECTS OF IODIDE AND THIOCYANATE IN CAUSING LOSS OF THE COLLECTED RADIOACTIVE IODIDE ARE SHOWN IN DETAIL. PATIENTS 5 THROUGH 14 HAD NOT RECEIVED OTHER ANTITHYROID MEDICATION BEFORE THE TEST. PATIENTS 2 THROUGH 4 ARE INCLUDED TO SHOW THE SPONTANEOUS COURSE OF ACCUMULATED RADIOACTIVE IODIDE WITHOUT THE INFLUENCE OF THIOCYANATE. PATIENT 3 HAD 2 GRAMS OF "TRIPLE BROMIDES" WITHOUT ANY EFFECT ON DISCHARGE OF ACCUMULATED IODIDE

	Maximum accumulation	Time of maximum after I^{131} administration	Dose of KSCN or KI	Course of I^{131} loss Time in hours after maximum, or KSCN or KI administration							Background**
				½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	
	c/s	Hours	Grams	c/s	c/s	c/s	c/s	c/s	c/s	c/s	c/s
1.	17	:47	1* (KSCN)	16	13	—	—	—	—	—	6–11
2.	50	1:04	0	48	45	40	36	32 (3:42)	—	—	5–6
3.	62	2:14	0	60	61	60	60 (2:38)	—	—	—	8–10
4.	82	1:45	0	—	81	78	79	77	71 (5:11)	—	—
	71	6:56	1 (KI)	33	21 (47 minutes)	—	—	—	—	—	—
				10 min.	20 min.	30 min.	1 hr.	2 hr.	3 hr.		
5.	60	1:34	1 (KSCN)	44	27	19	12	10 (1:30)	—	—	5
6.	102	2:54	1 (KSCN)	53	40	29	22	—	—	—	8
7.	143	2:20	1 (KI)	124	102	64	22	9	—	—	7
8.	175	3:40	1 (KSCN)	124	115	104	62	38	34 (2:38)	—	9
9.	70	1:52	2 (KSCN)	38	29	24	10	—	—	—	6–8
10.	79	1:16	1 (KSCN)	40	25	19	15	14	14	—	8
11.	90	1:18	1 (KSCN)	81	70	60	36	26 (1:49)	—	—	—
12.	90	1:10	1 (KSCN)	80	61	49	21	15	9	—	5
13.	95	2:23	1 (KSCN)	62	27	23	22	22 (1:33)	—	—	—
14.	69	1:19	1 (KSCN)	35	30	25	11	9	—	—	6–7

* This patient received an *additional* gram of potassium thiocyanate 33 minutes before the I^{131} was given.
** Obtained by counting over the upper anterior chest

with the first step in thyroid hormone formation. These drugs do not prevent the uptake of the iodide ion by the thyroid. Once bound to protein, thyroid iodine is no longer freely diffusible; it can no longer be discharged by thiocyanate or diluted out by ordinary iodide. Thus, the proportion of the accumulated radioactive iodine which leaves the thyroid under the influence of a standard dose of thiocyanate is an index of the degree of thyroid inhibition at the time of the procedure. At the end of the test the difference between the count over the thyroid region and the background represents radioactive iodine bound to protein during the period of the test. Presumably this iodine is available for the formation of thyroid hormone. These phenomena

can be directly utilized in determining the efficacy of antithyroid medication at any time after institution of therapy. By proper timing of the test in relation to the last dose, the duration of action of a single dose can be estimated.

In Figure 3 and Table 3 are shown examples of various degrees of inhibition of binding of accumulated radioactive iodine during therapy with antithyroid drugs. The significant findings here are the relative amounts of the accumulated iodine which failed to be discharged, and, hence, were presumably available for hormone synthesis. The absolute heights of the maxima were found to vary from time to time during treatment, but the factors involved in these changes are incompletely understood at present. The tests done after treatment in patient A (Table 3 and Figure 3) with propylthiouracil (300 milligrams per day) and mercaptoimidazole (40 and 60 milligrams per day) are good examples of the variations in degrees of inhibition of organic binding of iodine which may occur with different doses of antithyroid compounds.

Effects of Large Doses of Iodide and of Bromide Instead of Thiocyanate: If one gram of potassium iodide in solution were given during the test instead of thiocyanate, under like conditions of previous inhibition of hormone formation, the same distinctive pattern of discharge of acumulated radioactive iodine resulted (Figures 1 and 2). Bromide, however, failed to produce a similar effect in a dose of 2 grams of "triple bromides" (Figure 2).

Effects of Variations in the Administration of Thiocyanate and Iodide: Doubling the dose of potassium thiocyanate (2 grams in a single dose) in several instances accelerated somewhat the process of discharge of accumulated iodide, but it did not produce a detectably more complete loss of iodide from the thyroid gland (Figure 1). On the other hand, a dose of 0.1 grams of potassium thiocyanate produced an incomplete loss of iodide as demonstrated by a further discharge when a further dose of 1 gram was given. Similarly, the administration of only 10 milligrams of potassium iodide had no detectable effect in one subject. The ingestion of a second dose of 1.0 gram of potassium thiocyanate or potassium iodide during the one to two hours following the first dose of 1 gram of either occasioned a slight further discharge of iodide. These observations suggested that doses of 1 to 2 grams of potassium thiocyanate and 1 gram of potassium iodide were large enough for studies of this kind.

Determinations on the concentration of thiocyanate in the serum yielded values of from 3.3 to 9 mg./100 cc. (as potassium thiocyanate) during the interval of 14 to 63 minutes following ingestion of 1 gram of potassium thiocyanate. The data of Lavietes *et al.* (1936) showed that administration of 1 to 2 grams of thiocyanate consistently resulted in serum thiocyanate levels of 5 to 10 mg.%. A definite augmentation of effect was seen in one patient following administration

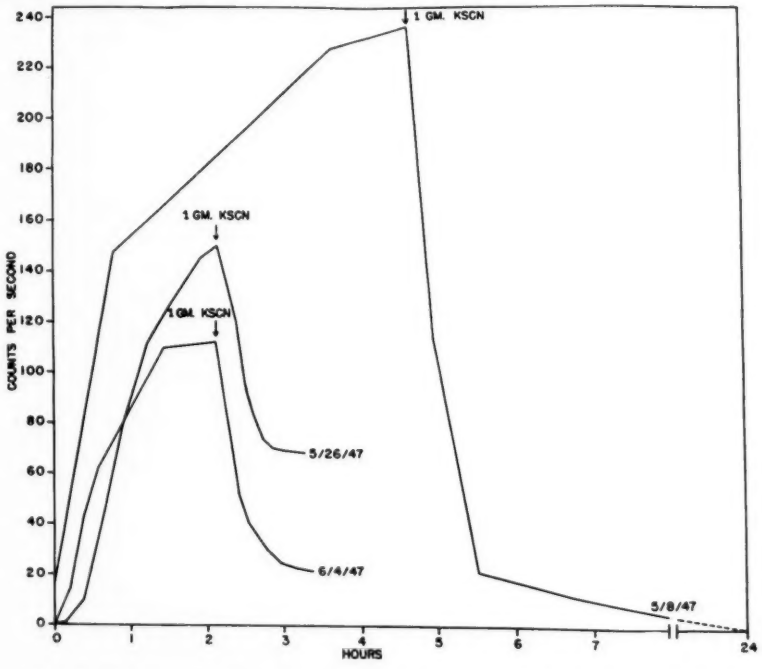

FIG. 3. The three graphs above represent radioactive iodide accumulations of patient A (Table 3) on three different occasions while she was receiving antithyroid drugs. The degree of inhibition of organic binding of collected radioactive iodine is indicated by the amount of iodide which was discharged by the potassium thiocyanate, relative to the amount accumulated before discharge began. On 5/8/47, with a dosage schedule of 20 milligrams of mercaptoimidazole every eight hours, there was only 2.3 per cent organic binding of collected iodide during the ten hours and twenty minutes' interval since the last dose (no further drug given during the test). While on propylthiouracil, 100 milligrams three times daily (test done 5/26/47), there was 45.3 per cent organic binding during the four hours and fifty-one minutes' period since the last dose of 100 milligrams. A third test (6/4/47), while the therapeutic schedule was 20 milligrams mercaptoimidazole every twelve hours, showed 21.3 per cent organic binding during the five hours and twenty minutes' period since the last dose of 20 milligrams. The values cited were all calculated from the level of discharge 59 minutes after the standard dose of potassium thiocyanate and hence are comparable, although they do not represent maximal loss in each instance. The control of the severe state of thyrotoxicosis as determined clinically could be correlated well with the above data. She seemed adequately treated with both 60 and 40 milligrams per day of mercaptoimidazole; she grew worse when 300 milligrams per day of propylthiouracil was being given.

of an additional gram of potassium thiocyanate when the serum level was 1.6 mg.%.

DISCUSSION

The rapid loss of radioactive iodine from the thyroid region produced by a large dose of non-radioactive potassium iodide suggests

TABLE 3. THE RESULTS OF TESTS FOR DETERMINATION OF THE EFFICIENCY OF INHIBITION OF ORGANIC BINDING OF IODIDE BY VARIOUS SCHEDULES OF TREATMENT WITH ANTITHYROID DRUGS IN TWELVE THYROTOXIC PATIENTS, AS DESCRIBED IN THE TEXT. THE COURSE OF THYROTOXICOSIS WHILE UNDER TREATMENT WAS ESTIMATED FROM CLINICAL AND OTHER LABORATORY DATA, AND WAS CORRELATED WITH THE RESULTS OF THE TESTS

Patient	Treatment	Maximal accumulation of I^{131}	Interval in hours after last regular dose of antithyroid drug			I^{131} Remaining after KSCN	% Uptake not discharged (organically bound)	Course under treatment
			When I^{131} given	When KSCN given	When test completed			
		c/s				c/s		
(A) 5/ 8/47	Mercaptoimidazole, 20 mg. every 8 hours for 8 days.	218.6	4:40	9:21	10:20 11:20	5 2	2.3* 0	Prompt improvement in an extremely toxic patient Dosage reduced to 20 mg. every 12 hours 5 days later.
5/26/47	Propylthiouracil, 100 mg. every 8 hours for 7 days.	150.4	1:35	3:52	4:51	68.2	45.3	Grew worse when propylthiouracil substituted for mercaptoimidazole. Mercaptoimidazole, 20 mg. every 12 hours started again day of test.
6/ 4/47	Mercaptoimidazole, 20 mg. every 12 hours for 9 days.	112.5	2:15	4:21	5:20 5:38	24.1 21.3	21.3* 19.0	Again improvement. It was repeatedly demonstrated that this dosage produced similar inhibition for as long as 9 hours. Clinical myxedema in 4½ months from beginning of treatment.
(B) 6/24/47	Mercaptoimidazole, 20 mg. every 12 hours for 14 days.	49.9	2:26	3:31	4:50	29.5	59.0	Moderate improvement but still toxic one month after this test; dosage then increased to 20 mg. mercaptoimidazole every 8 hours.
9/15/47	Mercaptoimidazole, 20 mg. every 8 hours for 7 weeks.	31.8	4:40	6:37	7:40	11.6	36.5	Early myxedema (total duration of treatment 3 months, 5 days).
(C) 8/13/47	Propylthiouracil, 50 mg. every 8 hours for 2½ months.	105.0	3:24	4:45	5:13	76.5	73.0	Still moderately toxic after 2½ months' treatment. Dosage increased to 100 mg. every 8 hours.
9/26/47	Propylthiouracil, 100 mg. every 8 hours for 44 days.	187.0	3:30	5:11	5:39 6:50	85.0 50.5	45.5* 27.0	Much improved on increased dosage.
(D) 5/ 2/47	Propylthiouracil, 100 mg. every 8 hours for 3 weeks.	34.8	3:46	8:00	8:42	29.8	86.0	No improvement in symptoms.
5/16/47	Same dose total 5 weeks.	46.0	4:33	7:15	8:44	42.0	91.5	Still no improvement. Started on mercaptoimidazole, 20 mg. every 8 hours with rapid change toward euthyroidism.

Patient	Treatment	Maximal accumulation of I^{131}	Interval in hours after last regular dose of antithyroid drug			I^{131} Remaining after KSCN	% Uptake not discharged (organically bound)	Course under treatment
			When I^{131} given	When KSCN given	When test completed			
		c/s				c/s		
(E) 5/23/47	Propylthiouracil, 100 mg. every 8 hours for 6 weeks.	180.0	1:05	5:20	8:00	27.5	15.3	Rapid improvement from beginning of treatment. Early hypothyroidism after 6 months' treatment.
(F) 5/17/47	Propylthiouracil, 100 mg. every 8 hours for 9 days	91.7	2:53	4:42	5:32	37.2	40.5	Treatment started with mercaptoimidazole, 4/13/37 to 5/8/47. Moderately better at this test. Further progress gradual. Hypothyroidism 6 months from institution of treatment.
(G) 6/23/47	Mercaptoimidazole, 20 mg. every 8 hours for 2 days.	194.5	1:39	3:55	4:30	51.0	26.2	Rapid and continued improvement. Dosage reduced to 20 mg. every 12 hours.
7/ 7/47	Mercaptoimidazole, 20 mg. every 12 hours for 2 weeks.	190.6	2:05	3:20	3:55 4:55	68.6 37.1	36.0* 19.5	Improvement maintained on smaller dose.
(H) 9/26/47	Mercaptoimidazole, 20 mg. every 8 hours for 7 weeks.	36.4	4:42	5:48	6:52	8.3	22.8	Rapid improvement from start of treatment.
(I) 10/ 8/47	Propylthiouracil, 100 mg. every 8 hours for 5 months.	130.0	4:15	5:38	6:42	65.0	50.0	Slow improvement. Goiter and blood cholesterol of 312 present at time of the test, but no clinical signs of myxedema were present.
(J) 9/15/47	Mercaptoimidazole, 20 mg. every 8 hours for 1 month.	94.5	2:14	4:29	6:15	22.8	24.2	Rapid improvement.
(K) 7/17/47	Propylthiouracil, 100 mg. every 8 hours for 3 months.	124.0	2:12	4:17	5:03	56.3	45.5	Improvement in symptoms during 3 months of treatment.
(L) 5/24/47	Propylthiouracil, 50 mg. every 8 hours for 4 months.	374.0	1:16	3:12	4:33	147.0	39.3	Improvement from 225 mg. daily maintained with 150 mg.

* Another test on same patient was terminated before maximal discharge had occurred. This value is for the concentration at the same interval after thiocyanate administration and allows direct comparison

that the iodine accumulated in the thyroid remains in the form of iodide ion when the gland is under the influence of an antithyroid compound. Apparently the thyroid gland possesses a mechanism by means of which the concentration of iodide ion in thyroid tissue can be maintained at a level many fold that normally present in the blood. In rats, VanderLaan and VanderLaan showed that this gradient of concentration was maintained despite a considerable increase in the total circulating iodine. They administered as much as 100 micrograms of potassium iodide without exceeding the capacity of the thryoid gland to maintain the same gradient. This dose corresponds to a dose of about 100 milligrams of potassium iodide to a human being. When a dose of 1 milligram was given to rats, corresponding to about 1 gram in man, only about one-tenth as high a gradient could be maintained. This probably indicates that there is a limit to the total amount of iodide that can be concentrated in the thyroid gland.

When a dose of 1 gram of potassium iodide is administered to man, the total iodide-ion in the body is greatly increased. The amount of iodide which the thyroid can accumulate is limited; hence, its ability to maintain a higher concentration in the thyroid than in the blood is markedly decreased in the presence of these large amounts of iodide in the body fluids. Since the specific activity of the iodide in the body fluids and the thyroid is the same, this sharp reduction in gradient is effective for the radioactive iodine atoms as well as the nonradioactive atoms; therefore, a much lower proportion of both remains in the thyroid, and the count over the thyroid drops rapidly as equilibrium is established.

The mechanism whereby thiocyanate acts is unexplained. One can only assume that it causes a breakdown in the selective mechanism for iodide concentration.

It is of some interest that virtually complete discharge of accumulated radioactive iodide was commonly observed in the normal individuals (i.e., count over thyroid was equal to or less than body background). This complete discharge did not occur in any of the thyrotoxic subjects with the exception of patient A (Figure 3 and Table 3, 5/8/47); the final levels were usually 2 to 24 counts per second higher than background. It was shown by VanderLaan and VanderLaan that a gradient of 250:1 for thyroid:serum iodide occurred in rats whose thyroids were hyperplastic from chronic propylthiouracil administration, whereas this gradient was only 25:1 in animals whose glands were normal. This ten-fold difference in concentration implies a still larger difference in total iodide, as the weights of the hyperplastic glands were several times greater than the normal weights. Thus, a difference of thirty-fold or more was observed when allowance was made for the larger size of the hyperplastic glands. Probably the effect of thiocyanate in one-to two-gram doses is to reduce greatly, but not to abolish, this gradient. If the gradient were

low in the beginning, as in normal subjects, thiocyanate might result in a near 1:1 thyroid:serum iodide ratio, whereas, if the gradient were very high, this same dose of thiocyanate would produce perhaps a 2–5:1 ratio.

There is an increased rate of production and secretion of thyroid hormone in thyrotoxicosis with a consequent increase in the turnover of iodine by the thyroid gland. Various components of this fundamental abnormality can be demonstrated with I^{131} in several different ways, including the method described in this paper. Another technique involves the administration of I^{131} without prior inhibition of hormone formation, the rate of accumulation being measured by counting over the thyroid region as described before. Rapid rates of uptake and large concentrations of I^{131} in the thyroid were encountered in previously untreated thyrotoxic patients, indicating that the major portion of the administered iodine entered the thyroid gland (Astwood and Stanley, 1947). The obvious explanation for this pattern of accumulation is that the I^{131} is entrapped as iodide by the thyroid cells which, being uninhibited, rapidly convert it into an organic form for storage in the follicles. Thus, the accumulation continuously increases because the I^{131} is not "dammed" in the thyroid cells in the form of iodide. However, the rate and extent of the accumulation in a few normal subjects and in some mild thyrotoxic patients fall within the same range. This overlapping detracts from the value of this method as a diagnostic procedure. A further disadvantage is that, in order to repeat the test during the course of treatment, therapy must be discontinued so that comparable results can be obtained, and no information as to the efficacy of treatment is obtainable.

A third variety of procedure involves the measurement of the I^{131} excreted in the urine during a period (usually 24 hours) following its administration (Rawson *et al.*, 1944, 1945; Keating *et al.*, 1947). The assumption is that the difference between the dose given and amount found in the urine reflects the quantity accumulated by the thyroid gland. The advantages of this type of test are that the estimations can be done at leisure, and precise measurements can be made of the quantity excreted. However, accurate urine collections are difficult unless the patient is under close observation in the hospital. A greater disadvantage is the relative paucity of information gained from the procedure; rapid changes in accumulation and discharge of iodine by the thyroid cannot be detected, and the method is inflexible.

If the rate of secretion of thyroid hormone could be measured, this would be a valuable aid in the diagnosis of thyrotoxicosis. Theoretically, this could be accomplished, using I^{131} as an indicator, by two methods:

In the first, the rate of loss of radioactive iodine from the thyroid gland could be determined. To do this the iodine could be permitted

to reach a maximal concentration and then observations could be made over several days while full doses of an antithyroid agent were given to prevent the reincorporation into the gland of I^{131} from catabolized thyroid hormone.

In the second method, the quantity of radioactive protein-bound iodine in the plasma could be measured following the administration of I^{131}. This method would require considerably larger, and perhaps dangerous, doses of tracer.

Both of these methods suffer the drawback of insufficient information to permit quantitative calculations. In order to calculate the actual rate of hormone synthesis or secretion, one would have to know the actual hormone content of the gland and the proportion of the radioactive iodine which was incorporated in the form of thyroid hormone.

The greatest single cause of error in these procedures for comparing the rates of turnover of thyroid iodine is the uncertainty in the previous iodine intake. As little as 3 milligrams of potassium iodide will greatly reduce the rate of uptake of a tracer dose of radioactive iodine by the uninhibited thyroid gland in a normal person. However, the quantity of iodide accumulated after a full dose of an antithyroid compound is much less affected by exogenous ordinary iodide. The finding of VanderLaan and VanderLaan that relatively large doses of ordinary iodide did not modify the thyroid to serum iodide ratio and the observation cited above that 10 milligrams of potassium iodide did not change the radioactive iodide concentration in man indicate that large differences in iodide intake would not give rise to error in this type of test.

The true significance of a large iodide accumulation by the thyroid gland is unknown. It is only known that a large iodide space develops in two conditions: in animals and man whose thyroids are made hyperplastic by prolonged antithyroid treatment, and in hyperthyroidism. Though hypertrophy and hyperplasia are common to these two conditions, it is not known that either of these states is directly related to the enlarged iodide space. Under most circumstances thyroid hypertrophy and hyperplasia are associated with depletion of colloid, a loss of total iodine, and an increase in vascularity. Further studies will be required to determine which aspect or concomitant of hyperplasia correlates with the enlarged capacity of the stimulated thyroid to concentrate iodide.

In any of the various tests discussed above, the administration of large amounts of iodine immediately prior to the test (either in the form of iodide or organically combined in compounds commonly used for x-ray visualization of the gallbladder, lung, or urinary tract) will often produce falsely negative results.

In following a number of patients during the course of therapy with various antithyroid compounds, the utility of the procedure

described herein for estimating the efficacy of treatment shortly after its inception was repeatedly demonstrated. If the level of I^{131} in the thyroid gland were very small after thiocyanate (less than 15 to 20 counts per second), indicating that nearly complete inhibition had occurred during the interval since the last dose of antithyroid drug, there was almost invariably a rapid response to treatment. This was particularly striking when the iodide uptake was very high, indicating that a large change had been effected in the originally great potential ability to produce hormone (patient A, Table 3 and Figure 3). This would be expressed both as a low percentage of the iodine used for hormone synthesis and a small absolute quantity remaining after thiocyanate. On the other hand, if the quantity of iodide accumulated in the thyroid were relatively low, as occasionally occurred while symptoms persisted (patient B, Table 3), and a small but significant amount remained after giving potassium thiocyanate, this prognosticated a poor response because only a small change in the rate of synthesis had been produced. This situation is expressed in Table 3 as a high percentage of organic binding and a low residual radio-iodine after thiocyanate. A large amount of I^{131} remaining after thiocyanate indicated a high level of organic binding, and hence, a suboptimal therapeutic response, even though this may represent moderate inhibition of synthesis originally extremely rapid (patient L, Table 3). Thus, one must take into account both the absolute level of iodine remaining in the thyroid gland after thiocyanate and the percentage inhibition of hormone synthesis which this level represents in order to anticipate properly the response to therapy.

Obviously, the test would be more informative if it were timed so that it ended when the next dose of drug was due; the inhibition of the thyroid would then be measured during the period of diminishing effectiveness and the result obtained in terms of minimal rather than maximal inhibition.

Previously, by means of tests on normal subjects, it was ascertained that 2-mercaptoimidazole was[5] a potent antithyroid agent, which was effective in inhibiting iodine accumulation by the thyroid for twenty-four hours or longer in doses of 25 milligrams. Confirmation of its efficacy in the treatment of thyrotoxicosis was obtained by the procedure described in this communication. Twenty patients have been treated with this material in dosage of 20 to 100 milligrams per day. Even in severely thyrotoxic subjects a dose of 20 milligrams sometimes produced nearly complete inhibition of hormone synthesis lasting as long as eleven hours. In other patients a dose of 20 milligrams was only partially effective. In one patient, myxedema occurred in $4\frac{1}{2}$ months with a dosage of 40 milligrams per day, and in another in three months with 60 milligrams per day.

For purpose of diagnosis, the method described in this communication seems to be superior to that based on total iodine accumulation

when the diagnosis of hyperthyroidism is under consideration. There is a greater difference between persons with normal thyroid glands and hyperthyroid patients in this test; it is probably much less sensitive to differences in the prior iodine intake than is the other method, and it has the additional advantage that it can be performed after antithyroid therapy has been started. Its major disadvantage is that it does not distinguish between hyperthyroidism and hyperplastic goiter from some other cause.

When the question of myxedema is being studied and in investigating certain types of goiter, measurement of the rate of total iodine uptake without previous administration of an antithyroid compound is preferable.

SUMMARY

After virtually complete inhibition of thyroid hormone synthesis by antithyroid medication, the human thyroid gland was still able to collect appreciable quantities of radioactive iodine believed to be in the form of iodide ion. After administration of thiocyanate or a large dose of ordinary iodide, this collected radioactive iodide was promptly, but usually not completely, discharged from the thyroid gland. This phenomenon of loss of radioactive iodine from the thyroid by dilution with large quantities of ordinary iodide adds to the evidence from animal experiments that the radioactive iodine collected under conditions of previous thyroid inhibition is in the form of iodide ion.

The pattern of accumulation of I^{131} in thyrotoxic patients was distinctive in that the extent of uptake was much larger than in normal individuals, and the rate of discharge was rapid after thiocyanate or ordinary iodide administration. These differences are great enough to provide an accurate means of detecting active thyroid hyperplasia, which is most commonly manifested as thyrotoxicosis.

The extent of loss of the collected radio iodide occasioned by a standard dose of thiocyanate was related to the degree of previous inhibition of hormone formation. Thus, the adequacy of a schedule of antithyroid therapy can be determined shortly after beginning treatment, and without interrupting the medication. The duration of action of single doses of an antithyroid compound is readily ascertainable.

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SPIRAL ARTERIES IN THE HUMAN OVARY

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OCCASIONALLY it happens that a scientific observation is made only to be forgotten and rediscovered after a long period of time. When it is rediscovered, it may be remembered because its significance is appreciated in the light of newly acquired knowledge. The original discovery in 1774 of the spiral arteries in the human endometrium by William Hunter is a case in point. It was not until Daron rediscovered these structures in the uterus of Macacus rhesus in 1936 that their relation to physiological events of the menstrual cycle began to be appreciated.

The recent observation that the arterial supply in the rabbit ovary is derived from a helical vessel of diminishing diameter lying along the hilus of the ovary (Reynolds, 1947a) is a second case in point. The recent literature on the subject of ovarian morphology and physiology contains no generally known reference to the existence and possible function of this structure until 1947 when it became the subject of several studies (Reynolds, 1947). This structure has been shown to play a special role in adaptation of the arterial supply to changes in size of the ovary, and in regulation of blood pressure within, and blood flow throughout, the ovary.

When these studies were under way, a chance observation was made of a publication by Belou of Buenos Aires in 1934, in which spiral arteries were described in a single instance in the human ovary. Belou described these by saying, "La ovarica (referring to ovarian blood vessel) permite detaller los pelotones de vasos helicinos del hilio del ovario." This observation has remained unnoted apparently, without attracting further attention of physiologists, anatomists or clinicians.

After studies of the nature of the spiral artery in the human ovary were begun at Cumberland Hospital, it was found, again by chance, that a description of the arterial arrangement in the human ovary was given by Farre in 1858. The details of this description fit so exactly the specimens shown below that it may be quoted verbatim. It

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reads, "The ovary derives its supply of blood chiefly from the ovarian (spermatic), but in part also from the uterine arteries. So free, indeed, is the communication between these vessels, that the organ may be equally well injected from either source. The communication is effected chiefly by means of a branch of the ovarian artery, which passes inwards to inosculate with a terminal branch of the uterine artery, this anastomotic branch being occasionally so large as to constitute the principal source of supply of the ovary. The terminal vessels are conducted to the lower border of the ovary between the

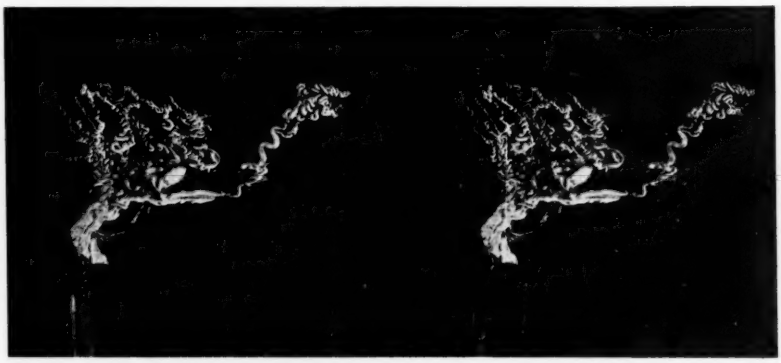

FIG. 1. Left ovary—Surgical—a 39 year old white woman, eighth pregnancy, four children—subtotal hysterectomy and bilateral salpingo oophorectomy. Ovarian artery injected. Stereoscopic view (×2). The vessels at the right have been moved out of their natural position.

folds of the posterior duplicature of the broad ligament, where they lie in parallel lines, and are readily distinguished by their tortuous or spiral form. Having entered the base of the organ, they spread out into those numerous ramifications which penetrate every part of the ovarian stroma, and give to this structure its peculiar fibrous aspect. From their extreme branches the blood is returned by the veins, which pass to the base of the organ, where they are very numerous. They form near the ovary and between the folds of the broad ligament, a plexus termed the ovarian or pampiniform plexus, the vessels of which communicate also with the uterine plexus. Valves are found in the ovarian veins only in exceptional cases."

Plastic casts of these vessels in three ovaries which were obtained by us are shown in Figures 1, 2 and 3.

This arrangement of the vessels in the human ovary was demonstrated in the manner previously described (3) as follows: The plastic, vinylacetate was injected into the vascular system of human ovaries. Surgical and post-mortem specimens were used. In some cases, in-

jection was made through the ovarian artery, and in others the uterine arteries were used. Equally good preparations were obtained in both instances, because of the free inosculation between the uterine and ovarian (spermatic) arteries in the hilus of the ovary to which Farre refers. The following procedure was used for injections:

FIG. 2. Left ovary—Post-mortem—a 39 year old white woman, twice pregnant with two living children—history of convulsions, hypertension and kidney disease. Uterine artery injected. Stereoscopic (×1½).

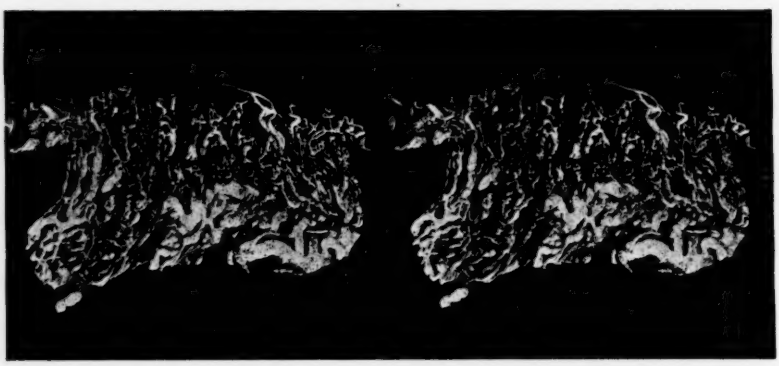

FIG. 3. Veins in the hilus of the ovary, showing the pampiniform plexus. Left ovary—post-mortem—a 53 year old white woman who was nephritic and diabetic. Ovarian vein injected. Stereoscopic (×2).

An 18 gauge hypodermic needle with blunted tip was inserted into the vessel to be injected. The tissue around it was tightly tied with a black silk ligature. The other arterial stump (either uterine or ovarian) was clamped. A small amount of acetone was injected and the cup of the needle filled. This precaution was taken to prevent premature

hardening of the plastic. Colored vinylite solution was then injected as rapidly and in as large quantity as possible. After fifteen minutes, the injected ovary was trimmed away from surrounding tissue and placed in the corrosion solution (HCl and pepsin) and incubated at 37°C. Depending upon the amount of sclerosis in the ovary, the resultant casts were digested free in ten to fifteen days. (The ovaries of a fetus required only twenty-four hours, as in the case of the rabbit's ovary.) Small, loosely adherent bits of tissue could be washed away under a gentle stream of tap water or by careful rotation in a beaker of water. Thus far, forty-five ovaries have been prepared in this way. Detailed studies of these in relation to the condition of the ovary at the time of injection will be reported elsewhere.

Inasmuch as we have acquired much knowledge concerning the physiology of the ovary in recent years, we may be expected to utilize this in studying the preparations obtained by this method. In this way, it may be possible to gain an insight into the etiology of certain pathological states of the ovary.

In the rabbit, for example, it has been found that the growth of the ovary resulting from gonadotropic stimulation causes orderly extension of the coils of the spiral artery in the ovary. In the presence of *corpus* hemorrhagicum cysts, however, there is distortion of the spiral artery during the period of follicular enlargement.

Preliminary study of the human preparations already available show marked differences in the vascularity of corpora lutea and of follicular cysts. Likewise, there are striking alterations in sclerotic ovaries, and the cast made from ovaries of a premature infant (7 month's gestation age) show a fascinating vascular tree upon which the complex spiral structures are destined to grow.

SUMMARY

Spiral arteries have been observed in the hilus of the human ovaries. These were demonstrated by means of plastic injection-corrosion preparations. These confirm in every detail the original and forgotten description of these structures by Farre in 1858.

Preliminary observations suggest a relationship between distortion of the spiral blood vessels and certain types of ovarian pathology.

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THE ROLE OF THE STEROID HORMONES IN THE RELAXATION OF THE SYMPHYSIS PUBIS OF THE GUINEA PIG[1]

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SINCE the discovery by Hisaw (1926) that the separation of the pubic bones in the guinea pig was under the control of a specific substance which was later called relaxin, several controversies have arisen. These controversies are concerned with the identity of this relaxative substance as distinct from the crystalline steroid hormones and with the role played by the steroids in the phenomenon of pubic relaxation.

Relaxin was first obtained from the blood of pregnant rabbits (Hisaw, 1926; Brouha and Simmonet, 1928; Tapfer and Haslhofer, 1935; Abramson, Hurwitt and Lesnick, 1937; Marder and Money, 1944). It has also been found in the blood of pregnant sows, dogs, cats, mares and in placentae of rabbits (Hisaw 1927 and 1929), blood of pregnant guinea pigs Hisaw (1927), Zarrow (1947), blood of pregnant women (Pommerenke, 1934; Abramson *et al.*, 1937), and corpora lutea of the sow (Hisaw, 1927; Brouha and Simmonet, 1928; Abramson *et al.*, 1937). Fevold *et al.* (1930a; 1930b; 1930c; 1932) prepared an active extract from the corpora lutea of sows containing 30 G.P. (guinea pig) units per mgm. of dry powder. This work was confirmed by Abramowitz *et al.* (1944) and extended by Albert, Money and Zarrow (1947) who were able to obtain an extract from the entire ovary of pregnant sows containing up to 500 G.P. units per mgm. of powder.

In 1931 de Fremery, Kober and Tausk secured pelvic separation with estrogen which was confirmed by Courrier (1931), Tapfer and Haslhofer (1935) and Dessau (1935). Furthermore, relaxation was reported after treatment with estradiol and progesterone (Fels, 1931; Möhle, 1933; Tapfer and Haslhofer, 1935; Haterius and Fugo, 1939). These observations led many investigators to question the validity of the relaxative substance as a distinct entity although Brouha and Desclin (1934) confirmed the original observation of Hisaw (1926) that relaxation in the estrinized guinea pig required only 6 to 8 hours

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after relaxin was administered whereas estrin alone required a number of days.

Hisaw *et al.* (1942 and 1944) confirmed the previous reports that progesterone caused pelvic relaxation, and pointed out that whereas relaxin produced its effect in 6 hours, progesterone required 72 to 96 hours. It was further indicated that the response to relaxin was identical in the castrated and castrated, hysterectomized guinea pig while progesterone was effective only in the castrated animal. In addition, it was shown that injection of estradiol and progesterone into rabbits with an intact uterus caused the formation of relaxin. In contrast to these experiments Fugo (1943) reported relaxation in castrated, hysterectomized guinea pigs after large doses of estradiol dipropionate and progesterone. Finally Courrier (1945) reported relaxation with (a) estrogen, (b) progesterone, (c) estradiol followed by progesterone, and (d) estradiol and progesterone given simultaneously. He obtained best results with both hormones given simultaneously but concluded that estrogen was the most important agent in relaxation and that it was unnecessary to postulate a new hormone.

Thus it is apparent that the situation with regard to the agents responsible for pelvic relaxation is most contradictory. The present investigation is an attempt to resolve the question of the number of substances responsible for separation of the pubic bones of the guinea pig and to determine whether pubic relaxation induced by the sex steroids is distinct from that induced by relaxin. It was also hoped that this study might shed additional light on the role of the uterus in the phenomenon of relaxation of the symphysis pubis.

MATERIAL AND METHODS

All guinea pigs were operated on at approximately 250 grams body weight and permitted to rest until they attained a body weight of 450 to 500 grams. The ovaries were removed under ether anesthesia by a lateral approach. The hysterectomies were performed by a similar lateral approach through which the ovaries and the upper part of the uterine horns were freed. This was followed by an abdominal incision through which the lower end of the uterus was freed, tied off posterior to the utero-cervical junction and removed. Thus in the castrated, hysterectomized guinea pig the ovaries, fallopian tubes, uterus and part of the vagina were removed.

The symphyses of the guinea pigs receiving injections of steroid hormones were palpated daily and recorded as negative, questionable, or positive, and in evaluating the data all questionable responses were regarded as negative. This is in accordance with the procedure followed in the routine assays for relaxin. At the conclusion of the treatment some of the animals were placed in metabolism cages and a 24 hour sample of urine collected under toluene for relaxin assay. Blood was obtained from all the animals by cardiac puncture and uteri removed, some of which were fixed in Bouin's for histological examination and others extracted for relaxin determinations.

Assays for relaxin content of urine, blood and tissues were carried out

with slight modifications according to the procedure of Abramowitz *et al.* (1944). The guinea pigs were primed with 1 μg of estradiol daily for three days and the extract to be tested was injected on the fourth day. The guinea pigs were palpated prior to the injection of the extract and only those animals with a tight pelvis were used. Six hours after injection of the extract the guinea pigs were palpated and results recorded as positive, negative or questionable. A guinea pig unit is defined as that amount of relaxin which produces definite relaxation of the symphysis pubis in two-thirds of a group of twelve or more castrated guinea pigs previously primed with 1 μg estradiol daily for three days.

Untreated blood serum was injected in a total volume of 1 ml. If a greater volume was required the serum was concentrated according to the procedure of Albert and Money (1946). When it was necessary to inject a small volume, the serum was always diluted to a constant volume of 1 ml. Urines were dialyzed in running water for 24 hours immediately after collection and when necessary concentrated by evaporation.

Tissue such as the ovary, uterus and vagina was ground up and extracted with 6 volumes of 3% HCl for 48 hours. The supernatant was removed by centrifugation and the residue re-extracted with 3 volumes of 3% HCl. The tissue was then discarded and the supernatants from the two centrifugations combined, neutralized to pH 7 to 7.4 and diluted for injection. If the volume was too great, it was reduced by precipitating with 5 volumes of acetone and redissolving the precipitate in saline at the desired volume. When it was necessary to store the extracts prior to carrying out the assays, the solutions were kept in the cold at an acid pH, under which conditions relaxin is stable.

The relaxin used in these experiments was prepared from whole, fresh sow ovaries (Albert, Money and Zarrow, 1947) and stored as a dry powder. Solutions of relaxin in normal saline were made up fresh as required. The steroid hormones,[3] estradiol, progesterone and desoxycorticosterone acetate were dissolved in sesame oil.

RESULTS

Estradiol and Progesterone

A comparison was made of the effect of estradiol and progesterone on the time required for relaxation of the symphysis pubis of castrated and castrated, hysterectomized guinea pigs. Both groups of animals received 0.5 mgm. of estradiol daily for the duration of the experiment. Starting on the eighth day of estradiol treatment the animals also received 1 mgm. of progesterone daily in addition to the estradiol.

The results obtained (Table 1) indicate that treatment with large doses of estradiol and progesterone produced relaxation of the pubic symphysis of both the castrated and the castrated, hysterectomized guinea pig without the addition of any exogenous relaxin. However, the average relaxation time for the castrated guinea pigs was 10.4 days

[3] The author is indebted to Schering Corporation, Bloomfield, New Jersey, for the progesterone, estradiol and desoxycorticosterone used in this study.

(range 10 to 11 days), while that for the castrated, hysterectomized group was 16.6 days (range 16 to 18 days), which is a significantly longer period. This difference is even more apparent if one compares the time required for pubic relaxation following the addition of progesterone to the treatment. The former group required 2.4 days while the latter group required 8.6 days. Assays for relaxin content of the blood and urine at the time of relaxation of the castrated guinea pigs showed 1 G.P. unit per ml. of blood serum and 1 G.P. unit per ml. of urine. The blood serum of the castrated, hysterectomized group

TABLE 1. TIME REQUIRED FOR PUBIC RELAXATION AND RELAXIN CONTENT OF BLOOD AND URINE OF CASTRATED AND CASTRATED, HYSTERECTOMIZED GUINEA PIGS AFTER TREATMENT WITH LARGE DOSES OF ESTRADIOL AND PROGESTERONE

G.P. no.	Treatment—daily Estradiol mgm.	Pro-gesterone mgm. from 8th day	Time for relaxation Total	Days following pro-gesterone treatment	Relaxin content G.P. uints/ml. Blood serum	Urine
Group 1. Castrated, Hysterectomized						
1	0.5	1.0	16	8		
2	0.5	1.0	18	10		
3	0.5	1.0	16	8		
4	0.5	1.0	16	8		
5	0.5	1.0	17	9		
		Average	16.6	8.6	Neg. at 4 ml.	Neg. at 2 ml.
Group 11, Castrated						
6	0.5	1.0	11	3		
7	0.5	1.0	10	2		
8	0.5	1.0	10	2		
9	0.5	1.0	11	3		
10	0.5	1.0	10	2		
		Average	10.4	2.4	1	1

also tested for relaxin content at the time of relaxation, was negative when tested at a level of 4 ml. of serum and urine was negative at a 2 ml. level.

In a second set of experiments castrated and castrated, hysterectomized guinea pigs were treated with (a) 10 μg of estradiol and (b) 10 μg of estradiol plus 1 or 2 mgm. of progesterone. The estradiol was injected daily from the start of the experiment until the animals relaxed while the progesterone treatment was started on the 11th day of estradiol treatment and continued daily until the end of the experiment.

Castrated guinea pigs treated with 10 μg of estradiol alone relaxed in an average time of 23.7 days and castrated, hysterectomized guinea pigs given the same treatment required an average time of 25.6 days for relaxation, a difference that is not significant. However, castrated guinea pigs treated with 10 μg of estradiol and 1 mgm. of progesterone showed 90% relaxation with an average time of 13.5 days. With 2

mgm. of progesterone all the animals relaxed in the average time of 13.0 days. In contrast with this, castrated, hysterectomized guinea pigs treated with 10 μg of estradiol and 1 mgm. of progesterone required 23.7 days for pubic relaxation, a definitely significant increase in the time required when compared with the castrated group (Table 2). Furthermore, a comparison of these results based on the time required for pubic relaxation following the start of progesterone treatment indicated that the castrated animals required only 3.0 to 3.5 days whereas the castrated, hysterectomized animals required 13.7

TABLE 2. RELAXATION OF THE SYMPHYSIS PUBIS AND RELAXIN CONTENT OF BLOOD, URINE, AND UTERI OF CASTRATED AND CASTRATED, HYSTERECTOMIZED GUINEA PIGS AFTER TREATMENT WITH MODERATE DOSES OF ESTRADIOL AND PROGESTERONE

No. of G.P.	Treatment—daily		Average relaxation time days		Relaxin content		
	Estradiol μg	Progesterone mgm.	Total	Following progesterone treatment	Blood serum G.P. units per ml.	Urine G.P.U per ml.	Uterus G.P.U. per gm.
Castrated							
9*	10	1 from day 11	13.5 (13–14)	3.5	0.5	0.3	10
10	10	2 from day 11	13.0 (12–14)	3	0.5	0.5	10
10	10	—	23.7 (16.31)	—	neg. at 4 ml.	neg. at 5 ml.	neg.
Castrated, Hysterectomized							
11	10	1 from day 11	23.7 (17–30)	13.7	neg. at 4 ml.	neg. at 8 ml.	—
10	10	—	25.6 (18–32)	—	neg. at 4 ml.	neg. at 4 ml.	—

* One guinea pig not included in the table required 22 days of treatment for pubic relaxation.

days. Finally it should be pointed out that the injection of 2 G.P. units of relaxin in either castrated or castrated, hysterectomized guinea pigs treated with estradiol for 10 days produced 100% pubic relaxation in 6 hours.

It is significant to note that relaxin was found in the blood, urine and uteri of castrated animals treated concurrently with both estradiol and progesterone whereas the castrated group given only estradiol and the castrated, hysterectomized animals given either estradiol or estradiol and progesterone showed no relaxin. The former group gave values of 0.5 G.P. units of relaxin per ml. of urine, and 10 G.P. units per gram equivalent of uterine tissue (Table 2).

Length of Progesterone Treatment

The treatment schedule used in the last experiment, wherein progesterone injections were started on the 11th day of estradiol treatment was an arbitrary one. Thus it was decided in the present study to determine whether starting the progesterone injections earlier than the 11th day of estradiol treatment would decrease the time required for pubic relaxation. A group of 5 castrated guinea pigs started with progesterone on the 6th day of estradiol treatment

showed a relaxation time of 13.2 days which is not significantly different from the results obtained when progesterone injections were started on the 11th day of estradiol treatment. Relaxin assays on the blood and urine indicated 0.3 G.P. units per ml. of serum and 0.5 G.P. units per ml. of urine. Castrated, hysterectomized guinea pigs treated in an identical fashion relaxed in the average time of 25.6 days and the blood and urine were negative for relaxin (Table 3). Again these results bring out in sharp contrast the difference in the time required for pubic relaxation between the castrated and the castrated, hysterectomized guinea pigs.

A second group of 5 castrated guinea pigs received 10 μg of estradiol and 2 mgm. of progesterone daily from the start of the experiment. This group required approximately 8 days for relaxation of the symphysis pubis, a significant decrease over the previous time requirements. The blood serum of these animals showed 0.5 G.P. units of relaxin per ml. (Table 3).

TABLE 3. THE EFFECT OF VARYING THE LENGTH OF TREATMENT OF PROGESTERONE ON THE TIME REQUIRED FOR RELAXATION OF THE SYMPHYSIS PUBIS OF THE CASTRATED AND CASTRATED, HYSTERECTOMIZED GUINEA PIG INJECTED WITH BOTH ESTRADIOL AND PROGESTERONE

No. of G.P.	Treatment—daily		Average relaxation time—days		Relaxin content G.P. unit/ml.	
	Estradiol μg	Progesterone mgm.	Total	Following progesterone treatment	Blood serum	Urine
Castrated						
5	10	2	(7–9)	8	0.5	—
5	10	2 from day 6	13.2 (11–14)	7.2	0.3	0.5
Castrated, Hysterectomized						
5	10	2 from day 6	25.6 (22–29)	19.6	neg.	neg.

Length of Estradiol Treatment

The purpose of this experiment was to secure data with regard to the necessity of continuing the estradiol treatment after progesterone injections were started in order to obtain pubic relaxation. A series of castrated guinea pigs were injected with 10 μg of estradiol daily for a total of 10 to 13 days and in all instances progesterone injections were started on the 11th day.

When the castrated guinea pigs were given 10 μg of estradiol for 13 days and 1 mgm. of progesterone from the 11th day, 90% showed pubic relaxation by the 14th day, *i.e.*, 3 days following progesterone treatment. However, when the estradiol was stopped on the 10th day, only 10% of the guinea pigs relaxed (Table 4).

A more thorough investigation was made using a similar estradiol injection schedule plus 2 mgm. of progesterone daily from the 11th day. Estradiol treatment for 13 or 14 days resulted in pubic relaxation

TABLE 4. THE PER CENT OF CASTRATED GUINEA PIGS SHOWING PUBIC RELAXATION AFTER TREATMENT WITH PROGESTERONE AND VARYING THE LENGTH OF TREATMENT WITH ESTRADIOL

No. of G.P.	Treatment—Daily		% of G.P. Relaxed
	Estradiol 10 μg daily for	Progesterone	
10	10 days	1 mgm. from day 11	10
10	13 days	1 mgm. from day 11	90
5	10 days	2 mgm. from day 11	0
5	11 days	2 mgm. from day 11	20
5	12 days	2 mgm. from day 11	80
10	13 days	2 mgm. from day 11	100
5	14 days	2 mgm. from day 11	100

in 100% of the animals within 14 days (2 to 3 days after the start of progesterone treatment). However, estradiol treatment for only 12 days plus a progesterone treatment similar to the above gave 80% relaxation by the 14th day. With a further decrease in length of estradiol treatment to 11 days only 20% of the animals relaxed, and finally when estradiol treatment was stopped on the 10th day and progesterone injections started on the 11th day (Fig. 1), no relaxation was obtained. Examination of blood and urine of the relaxed guinea pigs showed approximately 0.5 G.P. units of relaxin per ml. of blood serum and urine.

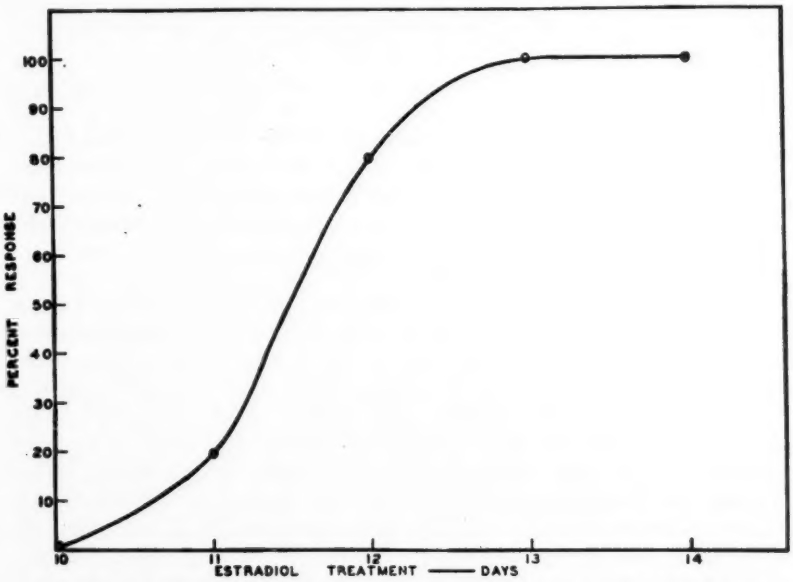

FIG. 1. Per cent of guinea pigs showing relaxation of the symphysis pubis, after treatment with estradiol and progesterone, plotted against length of estradiol treatment in days. The animals received 10 μg of estradiol daily and 2 mgm. of progesterone daily starting on the 11th day of estradiol treatment.

Estradiol and Desoxycorticosterone Acetate

The ability of desoxycorticosterone acetate and progesterone to act interchangeably under certain conditions made it seem worth while to investigate the effect of the former on the symphysis pubis of the guinea pig.

A group of 10 castrated guinea pigs were given 10 μg of estradiol daily for the duration of the experiment followed by 10 mgm. of desoxycorticosterone acetate daily from the 11th day of estradiol treatment. Relaxation of the pubic symphysis was obtained in 80%

TABLE 5. THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON RELAXATION OF THE SYMPHYSIS PUBIS OF GUINEA PIGS, TREATED WITH 10 μg OF ESTRADIOL DAILY THROUGHOUT THE EXPERIMENT AND WITH 10 MGM. OF DESOXYCORTICOSTERONE ACETATE DAILY FROM THE 11TH DAY OF ESTRADIOL INJECTIONS

G.P. No.	Time for relaxation days		Relaxin content G.P. units/ml.		G.P. units/gm. Uterus
	Total	Following DCA treatment	Blood serum	Urine	
72	13	3			
73	did not relax				
74	13	3			
75	13	3			
76	14	4	0.25	—	10
123	13	3			
124	did not relax				
125	13	3			
126	13	3			
127	13	3	0.33	0.33	10
Average	13.1	3.1	0.29	0.33	10

of the animals with an average relaxation time of 13.1 days or 3.1 days after the start of DCA treatment (Table 5). Assays on the blood and uteri indicated 0.25 to 0.33 G.P. units of relaxin per ml. of serum, 0.33 G.P. units per ml. of urine and 10 G.P. units per gram of uterine tissue.

DISCUSSION

Different investigators have indicated that three separate substances may cause pubic relaxation in the guinea pig. These substances are relaxin (Hisaw, 1926 and 1929; Brouha and Simmonet, 1928; Brouha and Desclin, 1934; Abramson, Hurwitt, and Lesnick, 1937), estrogen (de Fremery, Kober, and Tausk, 1931; Courrier, 1931; Brouha, 1931; Dessau, 1935; Tapfer and Haslhofer, 1935) and a conbination of estrogen and progesterone (Fels, 1931; Möhle, 1933; Tapfer and Haslhofer, 1935; Haterius and Fugo, 1939; Fugo, 1943; Hisaw, Zarrow, Money, Talmage, and Abramowitz, 1944). The fact that pubic relaxation may be produced by the sex steroids in addition to relaxin has not only tended to increase the confusion already present in this field, but has also led some investigators to doubt the existence of relaxin.

In 1934, Brouha and Desclin showed that relaxin produced pubic relaxation in a much shorter time than did the sex steroids. This was confirmed by Hisaw, *et al.* (1944) who indicated that while relaxin would produce pubic relaxation in 6 hours in the guinea pig, progesterone required 72 to 96 hours. Furthermore, relaxin was effective in both castrated and castrated, hysterectomized guinea pigs, whereas progesterone was effective only in castrated animals. In addition, these authors showed that progesterone caused the production of relaxin in rabbits with an intact uterus, and thus indicated that the necessity of an intact uterus for pubic relaxation by progesterone was additional evidence for the concept that progesterone and relaxin were separate entities. Fugo (1943) showed that massive doses of estradiol dipropionate plus progesterone caused pubic relaxation in the castrated, hysterectomized guinea pig and concluded the uterus was not essential for the relaxative effect of progesterone.

In the present investigation, the work of Fugo was repeated using the same injection schedule plus comparable amounts of estradiol and progesterone. It was found that the castrated, hysterectomized guinea pigs relaxed in the average time of 16.6 days which was similar to the results obtained by Fugo. However, the castrated animals relaxed in the average time of 10.4 days, a significantly shorter time. This difference is even more apparent when one compares the time required for pubic relaxation following the addition of progesterone to the treatment. The former group required 2.4 days while the latter group required 8.6 days. Furthermore relaxin assays of blood and urine were negative for the castrated, hysterectomized group, whereas the castrated animals showed approximately 1 G.P. unit per ml. of blood serum and 1 G.P. unit per ml. of urine. These results would seem to indicate that the uterus does play a significant role in the formation of relaxin, that relaxation produced by progesterone is mediated by the formation of relaxin in the body and differs from relaxation produced by estrogens. In view of the high doses of estradiol employed in the first experiment, the work was repeated with more physiological doses of estradiol and progesterone and a greater number of controls. It was found that castrated guinea pigs treated with estradiol and castrated, hysterectomized guinea pigs treated with estradiol alone or estradiol and progesterone relaxed in approximately the same time, namely in about 23 days. However, castrated animals treated with both estradiol and progesterone relaxed in approximately 13 days, a little more than half the time required by the other groups, and in addition relaxin was found in their blood, urine and uteri, whereas no relaxin was found in the castrated animals treated with estradiol or the castrated, hysterectomized animals treated with estradiol or estradiol and progesterone. Comparison of the time required for pubic relaxation following the addition of progesterone to the treatment, makes the difference in the time requirements more outstanding. The

castrated group required 3 to 3.5 days while the castrated, hysterectomized group required 13.7 days.

Thus it is possible to produce relaxation of the symphysis pubis of the castrated, hysterectomized guinea pig by either estrogen or a combination of estrogen and progesterone. However, the time required for relaxation in the castrated, hysterectomized guinea pig is the same regardless of whether estrogen is given alone or in conjunction with progesterone. This would seem to indicate that in the guinea pig lacking a uterus, estrogen is the significant factor and the progesterone is not necessary.

However, in the castrated guinea pig with an intact uterus, treatment with progesterone in addition to estradiol, both shortens the time required for relaxation almost in half when compared with an estrogen treated group and also results in the appearance of relaxin in the blood, urine and uterus. Thus it seems that in an estrin primed, guinea pig with an intact uterus, progesterone causes the production of relaxin which results in the relaxation of the symphysis pubis.

It must also be pointed out that the present studies confirm the previous reports that the effect of injected relaxin on the symphysis pubis is noticeable in a matter of hours and is equally apparent in both castrated and castrated, hysterectomized animals. Injection of 2 G.P. units of relaxin in guinea pigs treated for 10 days with 10 μg of estradiol produced relaxation in 6 hours.

From these results one may conclude that relaxation of the symphysis pubis of the guinea pig may take place by means of two different procedures: (1) prolonged treatment with estradiol which apparently has a direct effect on the symphysis; (2) treatment with relaxin—exogenous relaxin may be injected into an estrogen primed guinea pig or endogenous relaxin may be formed in the animals with an intact uterus after treatment with estradiol and progesterone.

The concept that pelvic relaxation as induced by estrogen differs from that brought about by relaxin is strongly supported by the histological studies made by Talmage (1947). He found that the symphysis pubis when relaxed by giving estrogen showed resorption of bone and proliferation of loose fibrous connective tissue whereas relaxin produced a breaking up and dissolution of collagenous fibers. He also found that the histological changes in the symphysis in response to progesterone were identical with those following the injection of relaxin.

Previous work on relaxation by the sex steroids involved pretreatment with estrogens followed by injections of both estrogen and progesterone simultaneously. Consequently it was decided to test the need for the simultaneous injection of estrogen and progesterone after the pretreatment was finished. The results obtained indicate that estrogen for 10 days followed by progesterone will not cause pubic relaxation but a positive response occurs when estrogen is given for 13 days and progesterone is started on the 11th day. The important

difference between these two experiments is that in the latter instance there is an overlapping of treatment with both hormones for a period of three days. When there was an overlapping of two days, 80% of the animals responded and for one day only 20%. Thus it would appear that a certain amount of overlapping of the treatment with estrogen and progesterone is necessary for relaxation. This may also indicate that either relaxin was produced after a 10 day treatment with estradiol followed by progesterone but the guinea pigs did not relax due to a loss in the priming effect of the estradiol by the 13th day, or that simultaneous treatment with both hormones is needed for the production of relaxin in the body.

The ability of desoxycorticosterone acetate to produce pubic relaxation and the formation of relaxin in the blood of estrogen primed guinea pigs is not surprising. Previous investigators have shown that progesterone and desoxycorticosterone have similar activities such as the progestational effect on the uterus (van Heuverswyn, Collins, Williams, and Gardner, 1939; Hohlweg; 1939; Robson, 1939), growth of the mammary glands (Mixner and Turner, 1942), prolongation of life in adrenalectomized animals (Emery and Schwabe, 1936; Gaunt and Hays, 1938; Schwabe and Emery, 1939), protection against cold (Zarrow, 1942) and withdrawal bleeding in monkeys (Hisaw, 1935; Speert, 1940). The present results confirm the findings of Emery (1946) that desoxycorticosterone acetate like progesterone can cause pubic relaxation but disagree as to the ratio of activity. Emery found that desoxycorticosterone acetate and progesterone were about equally active, however, he had pretreated his animals with massive doses of estrogen. The present investigation indicates that the ratio of activity of progesterone to desoxycorticosterone acetate is approximately 1 to 10 in the production of pubic relaxation in the castrated guinea pig and that desoxycorticosterone acetate can also cause the formation of relaxin in the castrated animal.

SUMMARY AND CONCLUSION

Relaxation of the symphysis pubis of the guinea pig may be produced by treatment with (a) estradiol, (b) estradiol and progesterone and (c) estradiol and relaxin. In the castrated, hysterectomized guinea pig progesterone is without effect whereas in the presence of a uterus treatment with estradiol and progesterone both shortens the time required for pelvic separation as compared with estradiol treatment alone and results in the formation of endogenous relaxin. Relaxin is effective in both castrated and castrated, hysterectomized guinea pigs which have been pretreated with estradiol, and produces relaxation in six hours. This is a much shorter time than that following treatment with estradiol alone or estradiol and progesterone. It is concluded that relaxation of the symphysis pubis of the guinea pig may take place by means of two different procedures: (1) prolonged treatment with estradiol which apparently has a direct effect on the symphysis and (2)

treatment with relaxin—exogenous relaxin may be injected into an estrogen primed guinea pig or endogenous relaxin may be formed after treatment with estradiol and progesterone in the presence of a uterus.

It is noted that optimum relaxation with the steroids is obtained when progesterone and estradiol are given simultaneously. Desoxycorticosterone acetate is approximately one tenth as active as progesterone in its ability to produce the formation of relaxin and pubic relaxation.

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THE INFLUENCE OF MIXTURES OF ANDROSTERONE AND DEHYDROISOANDROSTERONE ON BIOLOGICAL RESPONSE

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We (Saier, Grauer and Starkey, 1943) and others (Friedgood and Whidden, 1941) observed that the chromogenic properties of androsterone were not altered by the presence of dehydroisoandrosterone in the same solution when varying mixtures of these two biologically active components were studied colorimetrically. As a result of this it was decided to observe the effect of mixtures of these androgens when determined by biological assay. Androsterone is known to be more active biologically than dehydroisoandrosterone. These normally occur in urine in a proportion of from 95%+5% to 85%+15%. (Bauman and Metzger, 1940) (Callow and Callow, 1938). In pathological states, such as virilism caused by a tumor of the adrenal cortex, the amount of dehydroisoandrosterone may increase from the original 5 to 15% up to 40 to 70% of the excreted androgen (Callow and Callow, 1936). It is pertinent and at the same time appears paradoxical that as the less active fraction (dehydroisoandrosterone) is increased in amount the virilizing influence is enhanced. The question arose as to what effect this disproportion of the two hormones in solution will have on reactive tissue in the animal organism. In order to determine this the comb of the baby chick was selected as the target organ on which to observe the biological response of varying proportions of the two active androgens.

METHOD

In our preliminary studies were observed that the influence of light on the chicks' combs was an important factor in the weight response of this organ to the effect of androsterone. We found (Starkey, Grauer and Saier, 1940) that there were two variable factors that influenced the growth of the combs of the chicks; namely, the amount of androgenic material employed, and the intensity of the light to which the chicks were exposed. It therefore became evident that the light factor must be made constant and of adequate intensity. In order to achieve this the cages in which the chicks were kept

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during the entire course of the experiment were wired with incandescent light bulbs of equal intensity. These cages were kept in a room that was thermostatically controlled for temperature and from which all extraneous light sources were eliminated by blackening of the windows. The lights were fastened at the tops of the cages so that they were all equidistant from the chicks. All of the chicks, including the control groups were exposed to this light source for 12 hours of each day. It was found that the visible light rays were the important factor in the spectrum since a group of chicks that were exposed only to ultra violet irradiation did not show the comb weight increment observed in those that were exposed to the visible light rays. In our present study we, therefore, applied this knowledge of the effect of light to the chick-comb-weight method of Dorfman and Greulich (1937).

Crystalline androsterone, dehydroisoandrosterone, and mixtures of varying proportions of these compounds[1] were dissolved separately in sesame oil and in 95% alcohol. We demonstrated (Starkey, Grauer and Saier, 1943) that an inert solvent such as sesame oil was preferable to alcohol as a vehicle for the routine biological assay of the androgens. In this study both of these vehicles were used in order to obviate the possible modifying influence of the solvent on the androgens in solution, and in order to act as a check on each other. The solutions and their proportionate mixtures that were employed were made up as follows:

100% Androsterone	
95% Androsterone +	5% Dehydroisoandrosterone
75% Androsterone +	25% Dehydroisoandrosterone
60% Androsterone +	40% Dehydroisoandrosterone
50% Androsterone +	50% Dehydroisoandrosterone
40% Androsterone +	60% Dehydroisoandrosterone
25% Androsterone +	75% Dehydroisoandrosterone
	100% Dehydroisoandrosterone

These proportions were chosen in order to establish the curve responses that could be expected in normal and in known pathologic states. The 95% androsterone +5% dehydroisoandrosterone mixture was taken to approximate the proportion present in normal urine. The mixtures that fell in the 25%+75% group and the 75%+25% group were taken to include the reported proportions that are found in adrenal cortical carcinoma. The solutions were made up at the beginning of each 6 day "run of chicks" and were used throughout that period of treatment. Particular care was taken to prevent evaporation of the alcoholic solutions by keeping them tightly stoppered and under refrigeration when not in use.

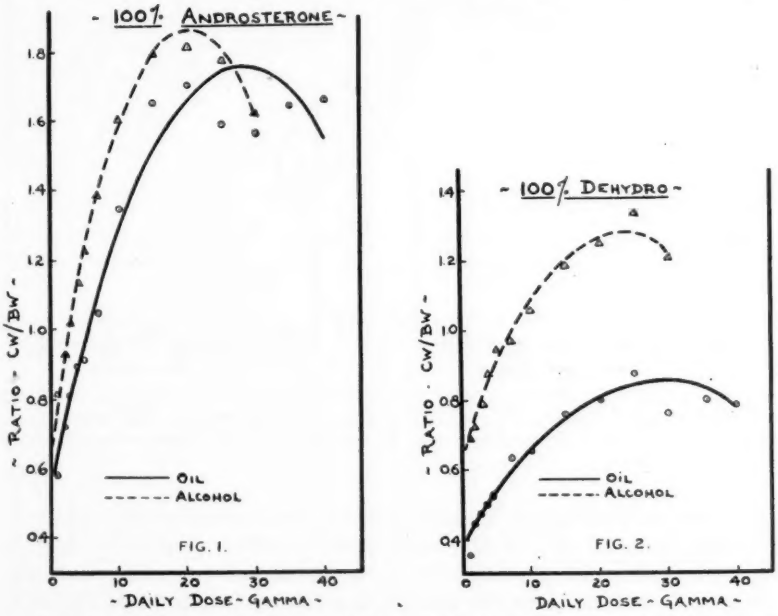
Exclusive of extensive preliminary work, 3,501 two-day old straight-run (unsexed) White Leghorn chicks were employed in this study. Of this number 118 were controls. All of the chicks were obtained from the same hatchery and were maintained under standard conditions of light and temperature inasmuch as we had demonstrated that variations in the intensity of light influenced the size and weight of the chicks' combs (Starkey, Grauer and Saier, 1940). The daily dose of the oily solution contained graduated doses of from 1 to 40 gamma and that of the alcoholic vehicle from 1 to 30 gamma of

[1] The androsterone and dehydroisoandrosterone were kindly supplied by Ciba Pharmaceutical Products through the courtesy of Dr. Ernst Oppenheimer.

the separate and combined androgens. Daily inunctions were made on the chicks' combs for six consecutive days in a constant amount of 0.01 cc. The chicks were anesthetized on the seventh day and their body weights were recorded. The combs were then removed and weighed. From the average weights of groups of 12 to 32 chicks on a given dose, the response was expressed as a comb weight/body weight ratio. From these data suitable (parabolic and log) curves were calculated by the method of least squares. Since the CW/BW ratio of the controls was fairly constant (0.30+0.02), they were not considered in calculating the response of the experimental animals.

RESULTS

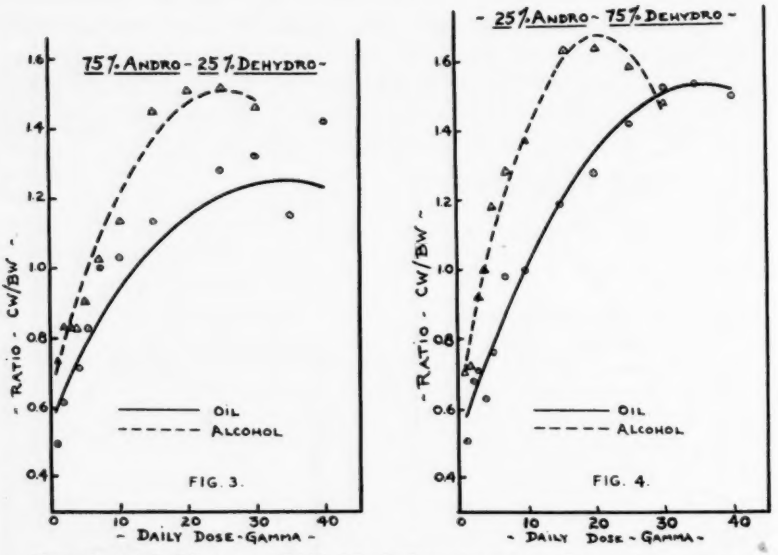
The individual solutions of 100% of androsterone and of 100% dehydroisoandrosterone gave, as was expected, biological responses which lay at the two extremes of the mixtures. We found the biological activity of crystalline androsterone in oil to be somewhat greater than twice that of the dehydroisoandrosterone (Figures 1 and 2). The



FIGS. 1 and 2. Curve responses of the individual solutions are established in oil and alcohol. The oily solution of androsterone is twice as active biologically as the dehydroisoandrosterone.

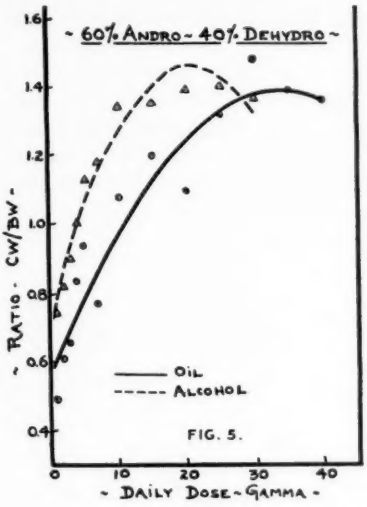
most significant findings lay between these two extremes. It is important to observe that the increase in the comb weights did not parallel the proportion of each androgen in the mixture. Thus, when 5% of dehydroisoandrosterone was mixed with 95% androsterone (Figure 7) the comb response approached that of 100% dehydroisoandrosterone

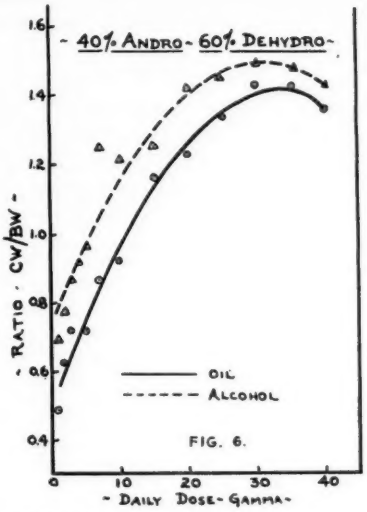
rather than that of androsterone. This surprising depressive action of only 5% dehydroisoandrosterone would lead one to expect the depressive biological effect of this androgen to increase in direct proportion to the amount present in the mixture. This, however, was not the case since the results that were obtained showed that the amount of the most biologically active substance did not control the order of response. Thus, when 25% of dehydroisoandrosterone was mixed with 75% of androsterone (Figure 3) the chicks' combs weights were slightly greater. This enhancement in biological response was further



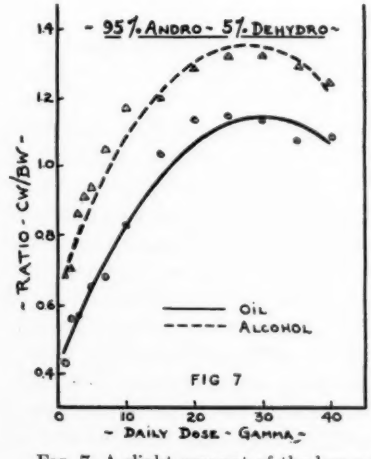
FIGS. 3 and 4. Mixture of 75% dehydroisoandrosterone (less active component) with 25% of androsterone gave the highest response in oily solution. Reversal of these proportions, gave a depressing response. As less active fraction was increased, tissue response increased.

observed as more dehydroisoandrosterone was added to the mixtures. Combinations of 60%+40% and of 40%+60% (Figures 5 and 6) showed practically no difference in response to each other, but did show an increment over the preceding mixtures in Figure 3. The mixture of 25% androsterone +75% dehydroisoandrosterone (Figure 4) gave further enhancement to the chick comb weight response. It was observed in all of our work, as evidenced by the curves, that the responsiveness of the chicks' comb to androgenic stimulation reaches a maximum point at a dose between 30 and 40 gamma, after which it declines. This was not the case when a mixture of equal parts of the two androgens were employed in an oily solution (Figure 8). At the 40 gamma dose, the chick comb weight response was still rising. This





FIGS. 5 and 6. Mixtures of 60%+40% and 40%+60% were almost identical in response.



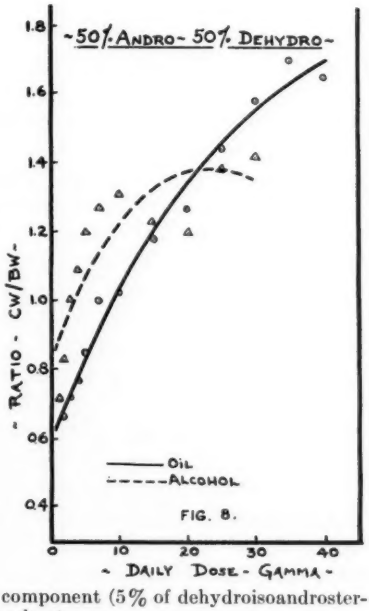
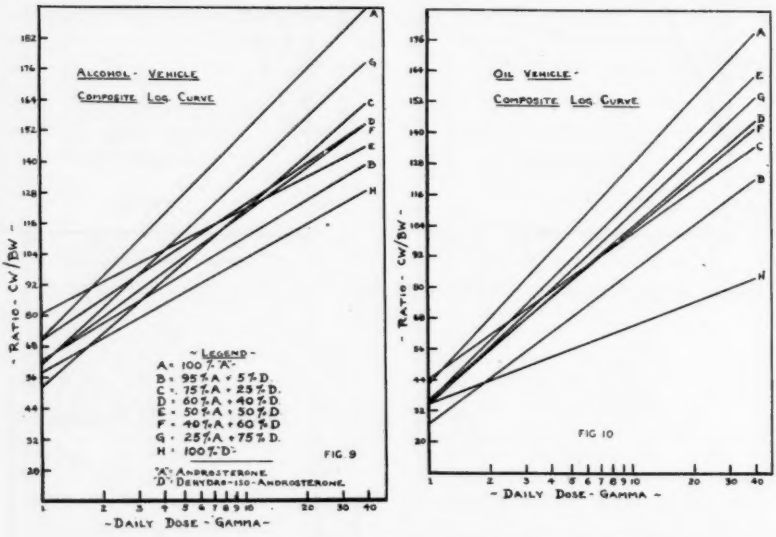


FIG. 7. A slight amount of the less active component (5% of dehydroisoandrosterone) depressed the biological activity of the androsterone.

FIG. 8. The oily solution of a 50–50 mixture gives a constantly rising response that can not be explained.

mixture was repeated on another "run" of chicks and was found to be correct.

The solutions of the androgens in alcohol gave greater enhancement of tissue response than those in oil, but we had observed that the oily solution was more consistently reliable. The order of response of the androgen mixtures in oil and in alcohol can be seen from the composite log curves plotted on semi-log graphs (Figures 9 and 10).


ALCOHOL - VEHICLE
COMPOSITE LOG CURVE
- RATIO - CW/BW -
- DAILY DOSE - GAMMA -
- LEGEND -
A = 100 % A
B = 95 % A + 5 % D
C = 75 % A + 25 % D.
D = 60 % A + 40 % D
E = 50 % A + 50 % D
F = 40 % A + 60 % D
G = 25 % A + 75 % D.
H = 100 % D
"A" = ANDROSTERONE.
"D" = DEHYDRO-ISO-ANDROSTERONE.
FIG. 9
OIL VEHICLE -
COMPOSITE LOG CURVE
- RATIO - CW/BW -
FIG 10
- DAILY DOSE - GAMMA -

FIGS. 9 and 10. Composite log curves showing order of response of the varying mixtures.

In order to further determine the order of response of these mixtures and in order to attempt to evaluate the unusual response of the 50–50 mixture when an oily solvent was used another approach was taken. An arbitrary ratio of CW/BW (ratio 1.28) was chosen and the daily dose of the mixture that was required to reach that ratio was read from the log graph. The order of response was determined by the least amount of androsterone in the mixture of the daily dose that reached the given ratio (Table 1). From this chart, it may be seen that the biologically most active mixture of these androgens is 25% androsterone +75% dehydroisoandrosterone and that the least active is that of 95% androsterone +5% dehydroisaondrosterone. The other mixtures fall in progression so that as the dehyroisoandrosterone is increased, at the expense of the androsterone, the mixtures become increasingly more potent. The 50% androsterone +50% dehydroisoandrosterone

in oily solution is seen to be irregular due to its peculiar activity. The interpretation of this response is not clear.

DISCUSSION

We previously observed that mixtures of crystalline androsterone and dehydroisoandrosterone in the same solution did not affect the chromogenic properties of the components in the mixture. The colorimetric calibration curves for the mixtures of these two subtsances fell

TABLE 1. MIXTURES OF ANDROSTERONE AND DEHYDROISOANDROSTERONE NECESSARY TO GIVE MAXIMUM BIOLOGICAL RESPONSE

Order of response determined by amt. of A	Alcohol vehicle				Oil vehicle			
	Mixtures of A & D*	Daily dose in gamma	Proportions of mixtures A	D	Mixtures of A & D*	Daily dose in gamma	Proportions of mixtures A	D
1	25 A–75 D	8.4	2.1	6.3	25 A–75 D	18	4.5	13.5
2	100 A	5.2	5.2	0	50 A–50 D	15	7.5	7.5
3	40 A–60 D	14	5.6	8.4	40 A–60 D	24.5	9.8	14.7
4	50 A–50 D	15	7.5	7.5	100 A	10	10.0	0
5	60 A–40 D	12.5	7.5	5.0	60 A–40 D	22	13.2	8.8
6	75 A–25 D	12.5	9.4	3.1	75 A–25 D	30	22.5	7.5
7	95 A– 5 D	24	22.8	1.2	95 A– 5 D	50	47.5	2.5
8	100 D	25.0	0	25.0	100 D—Reaches a ratio of only 0.84			

* A—Androsterone D—Dehydroisoandrosterone.

Note: Table was determined by the daily dose that each mixture required in order to reach $\frac{\text{comb-weight}}{\text{body-weight}}$ ratio of 1.28. Order of response was determined by the least amount of androsterone in the mixture of the daily dose that would reach this ratio.

between those constructed for the individual hormones and were found to be equal to the sum of the components. Our studies on the chick's comb indicated that this mathematical response did not prevail with biological material, and that potentiation appeared to exist in certain proportionate mixtures between androsterone and dehydroisoandrosterone in vivo. Dehydroisoandrosterone, while much less active than androsterone when used alone, became very active when combined with a small amount of androsterone. However, a small amount of dehydroisoandrosterone combined with androsterone depressed the normally enhanced activity of the androsterone, resulting in a much depressed response. The important implications of these findings become immediately apparent when we review the altered proportion of these substances in the pathologic state in the human as compared to the excretions as found normally. Callow and Callow (1940) found that the relative proportions of androsterone, hydroxyetiocholanone, and dehydroisoandrosterone in normal male urine were 1.6, 1.4 and 0.2 mg. per liter, respectively. In the normal female the same steroids were present, androsterone and hydroxyetiocholanone being about equal and dehydroisoandrosterone being present in 10 to 15% of the total. In the eunuch the proportions were altered to 0.5, 0.9 and 2.0 mg. per liter. Conversely, in adrenal virilism in the female the dehydroisoandrosterone is increased up to 40 to 70% of the

total. Thus, while the total steroids may be the same in the urine from two individuals, the proportions of the biologically active androgens may vary greatly and be associated with abnormalities in the human.

Separation of the total 17-ketosteroids into the alpha and beta fractions (Talbot, Butler and MacLachlan, 1940) (Bauman and Metzger, 1940) would tend to indicate by colorimetric methods the proportions of androsterone and of dehydroisoandrosterone that occur in a given specimen of urine. Other components in the biological material being assayed may have a modifying influence that would not be indicated by the colorimetric values. Thus, the beta fraction will probably contain biologically active isoandrosterone as well as dehydroisoandrosterone. In the light of our observations, the biological influence of isoandrosterone on the beta fraction can be speculated upon but would not be revealed by colorimetric fractionation.

This raises another important consideration. Biological assays are generally interpreted from a standard androsterone response curve which is plotted from pure androsterone. When biological material, such as extracted urine, is employed the differing proportions of the two active androgens will give a response that is at variance with a fixed curve derived only from androsterone. Consequently, it appears advisable to employ curves that are determined from mixtures of the two active androgens in proportions approximating the normal human rather than from a curve derived only from androsterone.

With the almost universal use of the colorimetric method for 17-ketosteroid determination, a better understanding of the relations of chromogenic properties to biological activity is pertinent. We assayed numerous normal and pathological urine specimens colorimetrically, using the M-dinitrobenzene method (Zimmerman reaction) for total 17-ketosteroids and attempted to confirm the results biologically by the capon and chick-comb-weight method, employing aliquot portions of the same urine. A parallel trend was obtained but attempts to correlate the results obtained by the two methods were not successful. This failure was possibly due to a lack of knowledge of the proportions of the biologically active androgens contained in the "total androgen" fraction. Pincus (9) indicated that a number of neutral ketosteroids acted as chromogens in the Zimmerman reaction. These substances, particularly non-alcoholic ketones, tended to cause an overestimate of the 17-ketosteroids. By the use of antimony trichloride he was able to identify androsterone and its isomers spectrophotometrically and could differentiate them from other substances that were indistinguishable with the Zimmerman reaction. This report indicates that a close approximation of the biologically active proportions of an unknown can be determined colorimetrically. Until this is fully accomplished, our observations emphasize the fact that colorimetric determinations, as generally performed, may give misleading results if

attempts are made to interpret them in relation to their possible biologically active proportions.

SUMMARY

It was found that mixtures of varying proportions of androsterone and dehydroisoandrosterone gave evidence of potentiation when the chick-comb-weight method of measurement was employed. Though pure androsterone is much more active biologically than dehydroisoandrosterone, it was found that a small dose of dehydroisoandrosterone (5%) had a marked biologically depressing action on androsterone. Paradoxically, as the amount of the biologically less active component (dehydroisoandrosterone) was increased in the mixture the biological response of the chicks' comb was enhanced. Contrary to anticipation, a mixture of 5% dehydroisoandrosterone +95% androsterone gave the most depressing response of the chicks' comb, while 75% dehydroisoandrosterone +25% androsterone gave the greatest response. Apparently the amount of androsterone in the mixture does not control the order of response of the biological material. The correlation of 17-ketosteroids with biological urinary assays for androgens is thus interfered with unless there is knowledge of the proportions of the active androgens present. The response of chicks' combs to the varying proportions of mixtures of biological active androgens was controlled by a constant light source.

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NOTES AND COMMENTS

PLASMA AND LIVER PROTEIN CONCENTRATIONS OF HYPOTHYROID RATS[1]

THIOURACIL and thiourea adminstration is followed by a significant increase in liver weight (Leblond and Hoff, 1944, May, Moseley and Forbes, 1946, Leathem and Seeley, 1947). Neutral fat and cholesterol levels do not change after thiourea feeding whereas liver glycogen has been reported as markedly increased (May, Moseley and Forbes, 1946) as well as decreased (Glock, 1945). The percentage of water and protein in these enlarged livers is normal so that an increase in liver protein per unit of body weight results (Leathem and Seeley, 1947). Leblond and Hoff (1944) indicate that liver atrophy due to thyroidectomy is not observed in the thiouracil induced hypothyroid state suggesting a unique action of thiouracil. This becomes more apparent in view of the reduction in food intake which follows either attempt to creat hypothyroidism as this in itself will decrease liver size and protein (Kosterlizt and Cramb, 1943) (Harrison and Long, 1945). In this report the effect of thiouracil feeding on the liver is compared with thyroidectomy and in each instance non-treated controls were pair fed.

Total plasma protein, plasma globulin and non-protein nitrogen increase in concentration while plasma albumin remains unchanged in male rats fed 0.5% thiouracil for 20–25 days (Leathem and Seeley, 1947). Rats that have been thyroidectomized exhibit similar plasma changes in comparison with ad libitum fed controls (Levin and Leathem, 1942) (Moore, Levin and Smelser, 1945) and are here compared with rats on controlled food intake.

Male rats of the Long-Evans strain were used at 150 days of age and kept in metabolism cages for measurement of daily food intake. Purina fox chow (22.2% protein) was fed ad libitum to thyroidectomized rats as well as to those receiving thiouracil[2] as 0.5 per cent of the diet. The experimental period was 20–25 days in duration after which time the rats were bled by cardiac puncture under light ether anesthesia and the plasma analyzed for non-protein nitrogen, total protein, albumin and globulin concentrations by methods reported previously (Leathem, 1945). The liver was dried to constant weight and was then ground to uniform consistency and analyzed for total nitrogen. Nitrogen values were converted to protein by use of the factor 6.25.

Figures 1 and 2 illustrate the effect of 0.5% thiouracil feeding and thyroidectomy on food intake as compared with a general average of 16 gms. per day per rat eating ad libitum. It is readily apparent that a return to normal food consumption was a slightly slower process for the operated rats. In both groups a loss in body weight was recorded as a reflection of the subnormal

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[1] This investigation was done under contract with the Office of Naval Research, Navy Department.

[2] Thiouracil (Deravet) was generously supplied by Dr. Mark Welsh of Lederle Laboratories, Pearl River, New York.

food intake, the pair fed controls showing body weight losses at least as great as the experimental animals.

Plasma protein concentrations revealed trends of similar nature in thiouracil fed and thyroidectomized rats. NPN, total plasma protein and

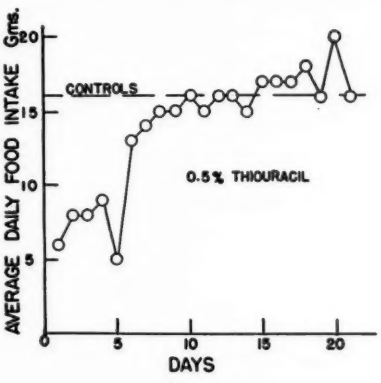

FIG. 1

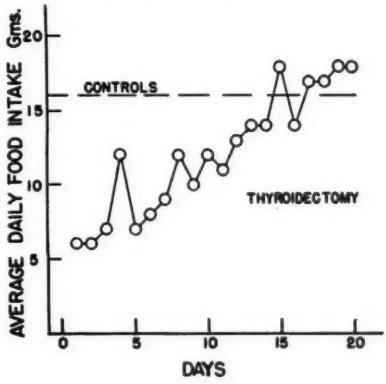

FIG. 2

plasma globulin concentrations increased while plasma albumin levels did not differ from those in pair fed controls. The hematocrit decreased in both hypothyroid states (Table 1).

TABLE 1. COMPARATIVE EFFECT OF THIOURACIL AND THYROIDECTOMY ON BODY WEIGHT AND PLASMA PROTEIN LEVELS OF RATS

No. of rats	Treatment	Body weight start	Body weight end	Hemato-crit	Non-protein N	Total protein	Albumin	Globulin
		gm.		%	mg./100 cc.	gm./100 cc.	gm./100 cc.	gm./100 cc.
8	thiouracil (0.5%)	330–320		43.6±0.5*	59.7±1.0	6.68±0.21	3.39±0.17	3.29±0.17
8	controls	336–320		45.9±0.5	52.0±1.9	5.75±0.19	3.11±0.15	2.63±0.10
9	thyroidectomy	315–294		41.1±0.7	50.0±2.0	6.50±0.21	2.91±0.17	3.59±0.14
9	controls	315–282		46.3±0.9	45.0±0.8	6.11±0.20	2.96±0.09	3.15±0.11

$$*\epsilon = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

The increase in liver size was again obtained with thiouracil and this liver tissue had a water and protein content comparable to those in control rats. Therefore, the total liver protein was significantly increased. Thyroidectomy, inducing a more rapid hypothyroidism than thiouracil, failed to increase liver size or to alter water or protein content (Table 2). Thus the effect of thiouracil on the liver would appear not be to due to hypothyroidism.

TABLE 2. COMPARATIVE EFFECT OF THIOURACIL AND THYROIDECTOMY ON THE LIVER OF RATS

No. of rats	Treatment	Weight gm.	Weight gm./100 gm. BW	Water %	Total protein gm.	Protein % dry wt.	Protein gm./100 gm. BW
8	thiouracil (0.5%)	15.2±0.6	4.2±0.2	71.0±0.3	2.93±0.13	66.9±1.2	0.886 (0.801–1.051)
8	controls	10.9±0.4	3.3±0.2	70.1±0.6	2.27±0.10	69.9±1.7	0.720 (0.631–0.790)
9	thyroidectomy	10.0±0.3	3.2±0.1	69.2±0.5	2.03±0.07	71.4±2.1	0.570 (0.610–0.825)
9	controls	9.5±0.5	3.4±0.2	70.3±0.2	2.02±0.06	74.0±2.4	0.710 (0.650–0.813)

SUMMARY

A rise in NPN, plasma globulin concentration and in total plasma protein occured in male rats 20 to 25 days after thyroidectomy or thiouracil feeding. Plasma albumin concentrations simulated those of pair fed controls. The hematocrit decreased.

Thiouracil induced an increase in liver weight while maintaining normal water and protein percentages. Total liver protein was increased. These liver effects were not duplicated in thyroidectomized rats.

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